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Chemotherapy of an Experimental *Fusobacterium (Sphaerophorus) necrophorum* Infection in Mice

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An experimental animal model for testing antibiotics in vivo against *Fusobacterium (Sphaerophorus) necrophorum* has been developed. It incorporates the subcutaneous injection of the bacteria into mice followed by intraperitoneal administration of the antibiotic at 24, 48, 72, and 96 h. Mean effective dose values are based on the number of survivors 21 days after challenge. Tetracycline was the most effective drug tested, with a mean effective dose of 5.0 mg/kg, compared with mean effective dose values of 11.1 for clindamycin, 11.8 for penicillin-G, and 52.9 for lincomycin.

Several of the nonsporing, gram-negative anaerobes are known to be of importance in diseases of man and animals. Although the in vitro susceptibility of many of these organisms to antibacterial agents has been determined, no animal model for testing the effects of such agents in vivo has been generally accepted. The major difficulty in developing an animal model arises from the lack of pathogenicity for laboratory animals of most members of the genera *Bacteroides* and *Fusobacterium*. *Fusobacterium (Sphaerophorus) necrophorum* is unusual in that it is pathogenic for rabbits and mice. The rabbit infection has previously been used for testing the effect of sulfonamides (11), but we are not aware of any use of the mouse for chemotherapy tests with this organism.

We used a strain of *F. necrophorum* that was especially virulent for mice to develop a model for testing antibiotics in vivo. The route of infection and the time and route of administration of antibiotics were systematically varied. Four antibiotics commonly used in the treatment of anaerobic infections were tested: penicillin, tetracycline, clindamycin, and lincomycin.

MATERIALS AND METHODS

Source of organisms. *F. necrophorum* VPI 6054-A (ATCC no. 27852) was originally isolated from a case of sheep foot rot. The original sample was furnished by the Dairy Science Department of Virginia Polytechnic Institute and State University.

Propagation of cultures. At the start of the series of experiments reported here, a single colony of strain 6054-A was picked into chopped-meat broth and lyophilized in a number of ampoules. One ampoule was opened for each experiment. The culture was grown in chopped-meat carbohydrate broth (8) and transferred daily two to four times prior to use.

Cell-washing procedure. A culture was grown overnight (15 to 18 h) to maximum turbidity in chopped-meat carbohydrate broth, from which meat particles had been removed prior to inoculation. The cells were sedimented anaerobically in rubber-stoppered tubes at 6,620 × *g* in a Sorvall SS-1 angle centrifuge. The stopper was removed under a stream of O_2-free CO_2, the supernatant fluid was removed, and the cells were resuspended in an equal volume of anaerobic dilution fluid (8). The cells were sedimented and washed three times in the same manner. Dilutions were then made anaerobically in the dilution fluid (8), and suspensions to be used for injections were transferred to serum bottles that were closed under CO_2.

Injection of bacteria. Immediately prior to injection of culture, plastic syringes were flushed several times with CO_2 and then filled from serum bottles. Subcutaneous injections were made beneath the loose skin of the groin on the animals' left side. All injections, whether subcutaneous or intraperitoneal, consisted of 0.1 ml.

Injection of antibiotics. All antibiotics were dissolved in distilled water and injected in 0.1-ml volumes. Solutions were made fresh for each injection. All procedures were aerobic. Subcutaneous injections were beneath the loose skin of the groin on the animals' left side.

Antibiotics. Penicillin-G, potassium, (1,600 U/mg) was from E. R. Squibb & Sons, New Brunswick, N.J.; tetracycline, HCl was purchased from Pfizer, Inc., New York. Lincomycin, HCl; clindamycin, HCl; and clindamycin, phosphate was from Upjohn Co., Kalamazoo, Mich.

Determination of minimal inhibitory concentration. Minimal inhibitory concentration (MIC) values were determined by a broth dilution procedure previously published (7). Clindamycin HCl was used for determination of MIC values to clindamycin, and

clindamycin phosphate was used for in vivo experiments in mice.

Viable counts. Viable cell counts were done by the roll tube procedure (8) in supplemented brain heart infusion agar medium (8).

Source of mice. Male Swiss white mice, 18 to 21 g (Flow Laboratories, Dublin, Va.), were used for all studies.

Anaerobic techniques and media. Roll tube procedures (8) were used for all culture manipulations, and all media were prepared prereduced (8).

Mean effective dose and mean lethal dose determinations. Twofold dilutions of either antibiotic or washed cells were used. A minimum of five concentrations was tested in duplicate in two separate experiments. Unless otherwise stated, 10 mice were tested at each concentration in each experiment, and death totals were calculated 14 days after injection of the bacteria. Statistical estimates were made both by the method of Reed and Muench (12) and by the probit method of Bliss (2). The 95% confidence limits were estimated by the method suggested by Miller et al. (10). Mean effective dose (ED_{50}) values are reported as milligrams per kilogram per injection.

Cultural examination of abscesses. Material from abscesses was streaked on blood agar plates and supplemented brain heart infusion roll tubes. Colonial appearance, hemolysis on blood agar plates, and the Gram stain reaction were criteria used for designation as a pure culture infection.

RESULTS

Infection. The mean lethal dose (LD_{50}) values for washed cells of *F. necrophorum* strain 6054-A are given in Table 1. The infection caused by intraperitoneal injection started as small abscesses either in the liver or any of the tissues in the peritoneal cavity. Such abscesses were barely visible 24 h after injection but reached sizes as large as 1 cm in diameter before death occurred. Each mouse normally had multiple abscesses. Examination of the abscesses revealed that this was a pure culture infection.

Subcutaneous infection could be caused with a smaller number of cells (Table 1), but the average length of life was longer than with the intraperitoneal infection (Fig. 1). One day after injection, abscesses of 2 to 3 mm could be found in the subcutaneous tissue. These subcutaneous abscesses were pure culture infections and remained localized in the subcutaneous area and thigh muscle until death. Subcutaneous injection of approximately 2.5×10^7 cells resulted in death of 97 of the 100 mice in 14 days. Intraperitoneal injection of twice as many cells resulted in death of only 88 of 100 animals in 14 days.

In vitro efficacy of antibiotics. The MIC values for the four antibiotics we tested are given in Table 2. Since agreement has not yet been reached on a standard method for determination of MIC values for anaerobes, we also determined the effect of the antibiotics by viable count experiments in supplemented brain heart infusion broth (Fig. 2). Penicillin-G was markedly bactericidal, and tetracycline also had some bactericidal activity at higher concentrations. Clindamycin and its parent compound, lincomycin, did not have significant bactericidal activity at these concentrations. Clindamycin was much more effective than the parent compound on a weight basis.

Chemotherapy. Initial experiments were done with the intraperitoneal infection. In these experiments, we injected the antibiotics 1 and 4 h after injection of the bacterial challenge. The results (Tables 3 and 4) were difficult to interpret both because of the 12% survival of animals that occurred without therapy and the extent of scatter of the data. The amount of antibiotic required to extend the survival time under these conditions was much larger than expected.

The next series of experiments made use of the subcutaneous model in which the background of survivors without therapy was negligible (3%). The results obtained with therapy of this infection at 1 and 4 h after challenge are given in Table 5. ED_{50} values were determined for these experiments. The amount of variation was less than with the intraperitoneal infection, but the amount of antibiotic required for therapy was still very large. Changing the therapy to

TABLE 1. *LD_{50} values for washed cell suspensions*

Route of injection	Method of calculation of LD_{50}	
	Reed and Muench[a]	Probit method of Bliss[b]
Intraperitoneal[c]	6.3×10^6 ($4.3\text{–}9.2 \times 10^6$)[d]	5.1×10^6 ($\pm 0.7 \times 10^6$)[e]
Subcutaneous[c]	2.4×10^6 ($1.8\text{–}3.1 \times 10^6$)[d]	2.7×10^6 ($\pm 0.6 \times 10^6$)[e]

[a] Reference 12.
[b] Reference 2.
[c] Intraperitoneal, total of 50 mice per group; subcutaneous, total of 20 mice per group.
[d] Estimate of the 95% confidence limits by the method suggested in Miller et al. (10).
[e] Standard deviation.

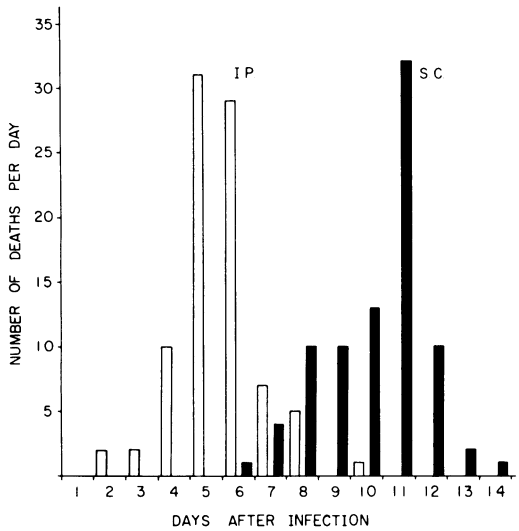

FIG. 1. *Number of mice, of 100, dying per day after intraperitoneal (I.P.) and subcutaneous (S.C.) inoculation of F. necrophorum.*

TABLE 2. *MIC values of antibiotics for F. necrophorum 6054-A*

Antibiotic	MIC value (μg/ml)
Tetracycline	0.2-0.4
Clindamycin	0.05-0.1
Lincomycin	0.4-0.8
Penicillin-G	0.05-0.1

injections at 24 and 27 h after challenge resulted in even less efficacy (Table 6). Longer term treatment consisting of injections of antibiotics 24, 48, 72, and 96 h after challenge greatly increased the effectiveness of the antibiotics tested (Tables 7 and 8). This course of therapy produced the most reproducible results, and the animals still alive at 3 weeks (Table 8) were usually completely free of infection.

DISCUSSION

The intraperitoneal route of infection resulted in shorter survival time than the subcutaneous route, but more mice survived the intraperitoneal challenge. This greater resistance to initiation of the intraperitoneal infection was also reflected in a higher LD_{50} value. Therapy of the intraperitoneal infection was possible, but there was considerable variation in results. This variation may have been due, in part, to the variation in site of the infection resulting from the intraperitoneal injection. Abscesses occurred in the liver, mesentery, pancreas, gut wall, peritoneal membrane, and gonads in various animals, and mice often had abscesses in several sites. In contrast, the subcutaneous injection produced a single abscess that remained localized in the subcutaneous tissue and the muscles of the thigh. This infection could be treated more reproducibly, and longer term therapy was possible because the course of the infection was longer.

The high ED_{50} values (>20 mg/kg) obtained when antibiotics were given only during the first day of infection were surprising to us. Such antibiotic regimes are commonly used for testing antibiotics on experimental infections caused by aerobic and facultatively aerobic pathogens. Such therapy may be prophylactic in nature in that the antibiotic probably pre-

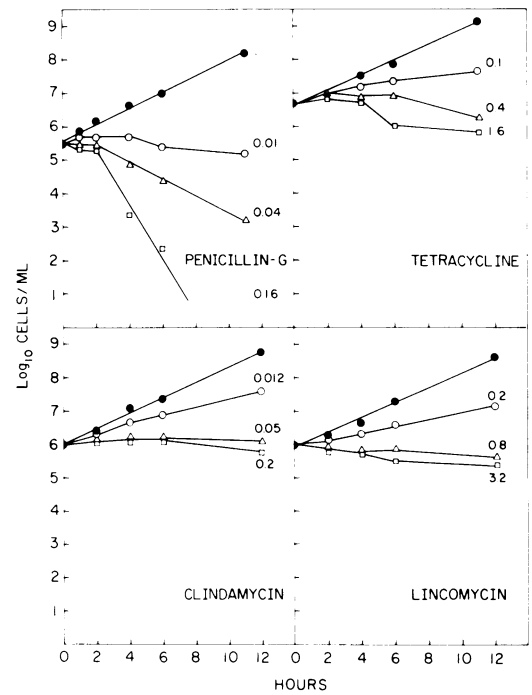

FIG. 2. *Effect of four antibiotics on the growth of F. necrophorum in brain heart infusion broth. ●, No antibiotic; numerals, micrograms per milliliter.*

TABLE 3. *Intraperitoneal infection treated by subcutaneous injection of antibiotics 1 and 4 h after challenge*

Antibiotics	Dose (mg/kg) per injection				
	125	63	31	16	8
Clindamycin	90[a]	60	25	25	40
Tetracycline		65	45	30	35
Penicillin-G	80	35	35	10	20

[a] Percentage survivors at 2 weeks with 20 mice per group.

TABLE 4. *Intraperitoneal infection treated by intraperitoneal injection of antibiotics 1 and 4 h after challenge*

Antibiotics	Dose (mg/kg) per injection				
	125	63	31	16	8
Clindamycin	60[a]	45	60	30	25
Tetracycline		85	90	30	20
Penicillin-G	45	40	60	55	25

[a] Percent survivors at 2 weeks with 20 mice per group.

TABLE 5. *ED_{50} values (mg/kg per injection) for treatment of the subcutaneous infection by intraperitoneal injections of antibiotics 1 and 4 h after challenge*

Antibiotic	Method of calculation of ED_{50}	
	Reed and Muench	Probit method of Bliss
Tetracycline	22.3 (11.0-42.3)[a]	23.4 (±5.4)[b]
Clindamycin	24.0 (21.4-27.0)	25.9 (±5.7)
Lincomycin	>125	>125
Penicillin-G	91.9 (41.0-202.0)	102.5 (±17.5)

[a] Estimate of 95% confidence limits (10).
[b] Standard deviation.

TABLE 6. *ED_{50} values (mg/kg per injection) for the treatment of the subcutaneous infection by intraperitoneal injections of antibiotics 24 and 27 h after challenge*

Antibiotic	Method of calculation of ED_{50}	
	Reed and Muench	Probit method of Bliss
Tetracycline	42.8 (36.8-52.3)[a]	53.0 (±13.5)[b]
Clindamycin	33.4 (28.2-39.6)	35.2 (±5.3)
Lincomycin	>125	>125
Penicillin-G	>125	>125

[a] Estimate of 95% confidence limits (10).
[b] Standard deviation.

vents the start of the infection. We do not have an explanation for the refractoriness of *F. necrophorum* to such an antibiotic regimen, but the ED_{50} values would be too high to allow comparison of the efficacy of antibiotics with less activity than those tested.

The treatment of the subcutaneous infection by antibiotic injections at 24, 48, 72, and 96 h was more effective than treatment confined to the 1st day of infection. The lower ED_{50} values should allow more accurate comparisons between antibiotics. The results also might be more meaningful for comparisons to actual clinical infections, because antibiotic therapy was not initiated until after the infection was established.

The time at which survivors were counted also influenced the results. Recording the survivors of the longer term therapy 14 days after the bacterial challenge was more a reflection of the ability of the antibiotics to prolong life than of complete cure of the mice, because many of these animals were very ill and died a short time later. Recording the results of the same experiment at 21 days was a better reflection of the ability of the drug to cure the animal of the infection, because almost all of the survivors in our tests were free of infection at this time. We found this procedure to be the most reproducible and recommend it to other investigators. Alterations in this procedure can yield different results in the comparison of antibiotics. For example, when results were recorded after 14 days, clindamycin was three times as effective as penicillin on a weight basis (Table 7), but the results of the same experiments recorded after 21 days showed equal activity for the two antibiotics (Table 8).

From the data presented in this report, we can conclude that all four of the antibiotics we

TABLE 7. *ED_{50} values (mg/kg per injection) for treatment of the subcutaneous infection by intraperitoneal injections of antibiotics 24, 48, 72, and 96 h after challenge (14-day survivors)*

Antibiotic	Method of calculation of ED_{50}	
	Reed and Muench	Probit method of Bliss
Tetracycline	1.7 (1.5-2.0)[a]	1.7 (±0.2)[b]
Clindamycin	2.8 (1.5-5.8)	2.6 (±0.3)
Lincomycin	11.4 (7.9-15.6)	11.4 (±2.7)
Penicillin-G	8.0 (6.5-10.0)	7.6 (±0.7)

[a] Estimate of 95% confidence limits (10).
[b] Standard deviation.

TABLE 8. *ED_{50} values (mg/kg per injection) for treatment of the subcutaneous infection by intraperitoneal injections of antibiotics 24, 48, 72, and 96 h after challenge (21-day survivors)*

Antibiotic	Method of calculation of ED_{50}	
	Reed and Muench	Probit method of Bliss
Tetracycline	5.0 (4.4-5.7)[a]	5.3 (±0.9)[b]
Clindamycin	11.1 (9.1-13.6)	11.0 (±2.1)
Lincomycin	52.9 (44.5-62.2)	56.3 (±7.3)
Penicillin-G	11.8 (11.5-12.2)	12.3 (±1.6)

[a] Estimate of 95% confidence limits (10).
[b] Standard deviation.

tested are capable of controlling this infection if given in adequate amounts. Lincomycin, in all cases, was the least active compound on a weight basis, but 7-chloro-lincomycin (clindamycin) was one of the most effective drugs. The disparity in the relative effect of these compounds on anaerobic bacteria has been documented (1), and clindamycin is effective in the treatment of human infection caused by nonsporing anaerobes (1, 4, 7). Tetracycline was also very effective in our experiments, and for many years it was the drug of choice for the treatment of anaerobic infections. The recent increase in the number of nonsporing anaerobes found to be resistant to tetracycline (15) has resulted in decreased use of tetracycline and increased use of clindamycin.

Because of the marked in vitro bactericidal activity of penicillin for *F. necrophorum*, we expected penicillin to be the most effective antibiotic in the treatment of this infection. Penicillin may not have as great a bactericidal activity on *F. necrophorum* in vivo, however, since *F. necrophorum* has been shown to be capable of formation of L-forms in the presence of penicillin (3, 14). Although we found penicillin to be less effective on a weight basis than the in vitro results would indicate, it was still almost as effective as clindamycin. In humans, however, Fellner and Dowell (5) reported that penicillin was ineffective in the treatment of two patients with *F. necrophorum* infections, and these authors also suggested that the production of L-forms might have been the cause of the lack of activity.

Although *F. necrophorum* does infect humans, it is primarily an animal pathogen. Liver abscesses in cattle cause an estimated loss of millions of dollars every year (13), and *F. necrophorum* is also involved in another major disease of cattle and sheep—foot rot (9). Tetracycline has been used for treatment of both of these diseases (6, 16).

Our use of *F. necrophorum* to initiate an experimental anaerobic infection in animals certainly is not new, but the previous work of which we are aware involved the use of larger animals, such as cattle (6), sheep (16), or rabbits (11), unsuitable for larger scale tests such as the present one. In addition, the exceptional virulence of this strain of *F. necrophorum* makes it possible to have death or survival as the sole criterion of the effectiveness of antibiotic therapy, instead of some other less simple criterion. We hope that the model we have used in this study will prove useful for investigators studying the efficacy of experimental antibiotics.

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