ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, June 1998, p. 1319–1322 Vol. 42, No. 6
0066-4804/98/$04.00+0

Minocycline and Cefotaxime in the Treatment of Experimental Murine *Vibrio vulnificus* Infection

YIN-CHING CHUANG,[1,2] WEN-CHIEN KO,[1,2] SHAN-TAIR WANG,[3] JIEN-WEI LIU,[1]†
CHIH-FENG KUO,[4] JIUNN-JONG WU,[5]* AND KUN-YEN HUANG[4]

Department of Internal Medicine, National Cheng Kung University Hospital,[1] and Departments of Medicine,[2] Public Health,[3] Microbiology and Immunology,[4] and Medical Technology,[5] National Cheng Kung University Medical College, Tainan, Taiwan

Received 8 September 1997/Returned for modification 18 December 1997/Accepted 23 March 1998

We conducted an in vivo study with the mouse model of *Vibrio vulnificus* infection to evaluate the efficacies of therapy with minocycline or cefotaxime alone and in combination. *V. vulnificus* was introduced subcutaneously into the area over the right thigh. The inoculum size ranged from 1.0×10^3 to 1.2×10^8 CFU from experiment to experiment but was constant for all animals in the same experiment. Antibiotics were given intraperitoneally 2 h after the bacteria were inoculated. In experiments 1 to 4, the standard dose for humans was used to treat the infection, while in experiment 5, five times the standard dose for humans was used to treat the infection. In experiment 1, with a small inoculum of 5×10^3 CFU, all mice in the saline-treated control group and the cefotaxime-, minocycline-, and combined antibiotic-treated groups survived. In experiment 2, with a moderate inoculum of 1.2×10^5 CFU, all the mice in the three antibiotic-treated groups survived, while only two of nine mice in the control group survived. In experiment 3, with a large inoculum of 8.0×10^7 CFU, six of nine mice in the combined antibiotic-treated group survived, while only one of nine mice in the cefotaxime-treated group and none of the mice in the control and minocycline-treated groups survived. In experiment 4, with a large inoculum of 1.2×10^8 CFU, 8 of 20 mice in the combined antibiotic-treated group survived, while none of the 20 mice in the control group, the group treated with cefotaxime alone, and the group treated with minocycline alone survived. In experiment 5, in which mice were infected with a large inoculum of 6.6×10^7 CFU and treated with five times the standard human dose of antibiotics, 10 of 12 mice in the combined antibiotic-treated group survived, while only 4 of 12 mice in the minocycline-treated group, 1 of 12 mice in the cefotaxime-treated group, and none of the mice in the control group survived. In experiments 3 to 5, the difference in the survival rates between the combined antibiotic-treated and minocycline-treated groups was statistically significant ($P < 0.05$). These results indicate that combination therapy with cefotaxime and minocycline is distinctly more advantageous than therapy with the single antibiotic regimen for the treatment of severe experimental *V. vulnificus* infections.

Vibrio vulnificus, a highly virulent halophilic gram-negative bacillus, is associated with a pathology in humans which may involve many organs and tissues (1, 15, 16, 21). There are two rapidly progressive clinical courses of *V. vulnificus* infection (1, 19): (i) primary bacteremia, which presumably follows ingestion of the agent, is often seen in patients with preexisting liver diseases, and (ii) wound infection, which begins as cellulitis following direct inoculation of the organism and which may result in tissue necrosis and secondary bacteremia, often occurs in previously healthy persons (1, 19). Cases of *V. vulnificus* infections have been reported from many areas of the world (1, 2, 19, 21). In Taiwan there has recently been a dramatic increase in the number of reported cases of infection due to this organism (4–9, 24). The cases of primary bacteremia are often complicated by severe soft-tissue infections such as necrotizing fasciitis and myonecrosis coupled with hemorrhagic bulla formation on the skin (8). The mortality rate among patients with primary septicemia in our series and those reported by others exceeds 50%, and death mostly occurs within 48 h of hospitalization (8, 17). About 40% of patients with wound infections have positive blood cultures, and the mortality rate among such patients is 25% (8).

The facts that many patients first seek medical treatment when they are already in the advanced stage of the disease and that this disease often runs a fulminant course make it crucially important that the antimicrobial agent initially chosen for therapy be correct if the treatment is to be successful. Regrettably, several issues regarding the chemotherapy for *V. vulnificus* infection remain unsettled. Most isolates of *V. vulnificus* are sensitive in vitro to a variety of antibiotics (3, 10, 14, 20). As a result, antibiotics recommended on the basis of in vitro susceptibility tests vary widely from study to study (12, 20, 23), but none of them has provided truly satisfactory therapeutic results. Among them, tetracycline has been the most highly recommended, but this recommendation was based on the results of a single study with animals in which the antibiotic was used alone (3). On the other hand, our own clinical experiences indicate that the broad-spectrum cephalosporins may be a good choice for patients with severe cases of infection (8, 9). Our previous time-kill study also showed that the combination of cefotaxime and minocycline produced a synergistic inhibitory effect against *V. vulnificus* that persisted for at least 48 h (10). In order to determine whether our in vitro observations are also reproducible in vivo, we conducted a study with the murine model to determine the role of antimicrobial agents in the treatment of *V. vulnificus* infections of different severities.

* Corresponding author. Mailing address: Department of Medical Technology, National Cheng Kung University Medical College. No. 1, University Rd., Tainan, Taiwan. Phone: 886-6-2353535, ext. 5775. Fax: 886-6-2363956. E-mail: jjWu@mail.ncku.edu.tw.

† Present address: Division of Infectious Diseases, Department of Internal Medicine, Chang Gung Memorial Hospital-Kaohsiung, Kaohsiung, Taiwan.

Such a study may usher in a new strategy for the clinical management of severe *V. vulnificus* disease.

MATERIALS AND METHODS

Bacterial strains. The clinical isolate *V. vulnificus* NCKUH No. 71 was used throughout the study. The organism was stored at −70°C in Luria-Bertani broth (Difco Laboratories, Detroit, Mich.) before being cultured on nutrient agar (Difco Laboratories) with 3% sodium chloride. Bacteria grown in the agar medium were maintained at room temperature, from which the inoculation suspension was prepared in Mueller-Hinton broth (Difco Laboratories). It was incubated overnight at 35°C in a water-bath shaker. The bacteria, which were further transferred to another Mueller-Hinton broth at a 1:50 dilution and which were incubated for 3 to 4 h under the same condition, were used for the study. They were collected by centrifugation, and the pellet was resuspended in 0.85% saline. The bacterial suspension was adjusted to the appropriate numbers of CFU per milliliter by turbidimetry with saline, and this was confirmed by the subsequent growth of the concurrent culture on spread plates.

Mice. Female inbred BALB/c mice (Animal Center, National Cheng Kung University Medical College, Tainan, Taiwan) weighing, on average, 20 g (age, 5 to 6 weeks) were used throughout the study.

Antimicrobial agents. The marketed parenteral forms of cefotaxime and minocycline used in the in vivo experiments were provided by Hoechst, Taiwan Co., Ltd., and Lederle, Parenterals, Inc., Carolina, Puerto Rico, respectively. The antibiotics were freshly diluted in sterile 0.85% saline on the morning of the experiment and were delivered in sterile disposable plastic syringes.

Preliminary study. *V. vulnificus* was injected subcutaneously (s.c.) in the area over the right thigh to groups of eight mice each, and the numbers of surviving mice were recorded every 6 h. In an attempt to define the severity and natural course of *V. vulnificus* infection in the mice, we randomly chose 10^3, 10^4, 10^5, 10^6, 10^7, and 10^8 CFU as the initial inoculum sizes.

Groups of two mice each were killed, their chests were opened, and blood samples for aerobic culture were withdrawn by cardiac tapping at 30, 60, 90, and 120 min after the injection of 10^8 CFU of bacteria.

General experimental design. According to the results of a preliminary study, we arbitrarily chose 5×10^3 CFU, 1.2×10^5 CFU, and 8.0×10^7, 1.2×10^8, and 6.6×10^7 CFU as initial inocula to represent mild, moderate, and severe infections, respectively. In five separate experiments, 5×10^3, 1.2×10^5, 8.0×10^7, 1.2×10^8, or 6.6×10^7 CFU of *V. vulnificus* was injected s.c. into the area over the right thigh of each mouse. In each experiment, there were four groups, including a control group and groups treated with cefotaxime, minocycline, and cefotaxime and minocycline in combination, with 9 to 20 mice in each group. The inoculum size was the same for the four groups of mice in every experiment. Cefotaxime or minocycline was given intraperitoneally in a 0.1-ml volume, beginning 2 h after the animal was infected, with the former drug being given every 6 h and the latter drug being given every 12 h thereafter, whereas control animals received 0.1 ml of sterile 0.85% saline every 6 h. The dose of antibiotics was determined according to the recommendations of the pharmaceutical companies, i.e., 30 mg of cefotaxime per kg of body weight every 6 h and a loading dose of 4 mg of minocycline per kg followed by a maintenance dose of 2 mg of minocycline per kg every 12 h. The antibiotics were given for a total of 42 h. In experiment 5, five times the standard dose for humans was used to treat the infection. The numbers of surviving mice were recorded at 6-h intervals after the initial treatment, and the recording of the numbers of surviving mice ended at 120 h. Survival means that at the time of assessment the mouse was still breathing, even if it was moribund.

Data analysis. The numbers and the percentages of surviving mice in the different treatment and control groups were recorded at 6-h intervals after the initial treatment. The percentages in the various groups at each of the designated observational times were compared by using the Fisher-Freeman-Halton statistic (13). This statistic is a generalization of the well-known Fisher's exact test for the analysis of two-by-two contingency tables to those with multiple rows and columns. When a P value was less than 0.05, post-hoc multiple comparisons by a Scheffe-type procedure (18) were made.

RESULTS

All the mice infected with 10^3 CFU survived for more than 120 h (Table 1). With 10^4 and 10^5 CFU as the initial inoculum, the mice died within 48 and 24 h, respectively. When the inoculum was increased to 10^6 or 10^7 CFU, all the mice died within 18 h. All the mice died within 6 h without exception when they were infected with 10^8 CFU of *V. vulnificus*. *V. vulnificus* grew from all blood samples taken at 30, 60, 90, and 120 min.

The numbers of mice which survived s.c. infection with *V. vulnificus* were recorded at 6-h intervals following antibiotic treatment, and the survival rate was calculated (Table 2). In experiment 1, with a small inoculum of 5.0×10^3 CFU, all the mice in both the control and the three different treatment groups survived. In experiment 2, with an inoculum of 1.2×10^5 CFU, seven of nine mice in the control group died within 24 h, while all the mice in the three treatment groups survived to the end of the experiment. In experiment 3, with an initial inoculum of 8.0×10^7 CFU, all mice in the control group died within 24 h, and eight of nine, four of nine, and three of nine in the cefotaxime, minocycline, and combination groups, respectively, died within 24 h. At 60 h, among five mice in the minocycline group, two mice developed focal black lesions over the entire right limb, two mice developed these lesions only over the distal limb, and one mouse developed these lesions only over the distal phalanges, while in the combination group (six mice) and the cefotaxime group (one mouse), save for local lesions at the site of injection of *V. vulnificus*, none of the mice developed lesions similar to those described above. All five mice in the minocycline group surviving at 60 h died 24 h later, while those in the cefotaxime and the combination groups stayed alive for more than 7 days after the discontinuation of the antibiotics. However, the only surviving mouse in the cefotaxime group developed limb necrosis 36 h after the antibiotic was discontinued. All surviving mice were limping, but the mice in the combination group were distinctly more active than those in the minocycline group. The survival rates recorded at the end of the experiment were 67 and 0% for the combined antibiotic and minocycline groups, respectively. The mice in the combination group did significantly better than the mice in either the cefotaxime or the minocycline group ($P < 0.05$). In experiment 4, with an initial inoculum of 1.2×10^8 CFU, all mice in the control group died within 6 h, all mice in the cefotaxime group died within 72 h, and all mice in the minocycline group died within 96 h, while 8 of the 12 mice in the combined therapy group survived. In experiment 5, with an initial inoculum of 6.6×10^7 CFU, all mice in the control group died within 12 h, while 1 of 12, 4 of 12, and 10 of 12 mice in the cefotaxime, minocycline, and combined groups, respectively, survived. In experiment 1, an inoculum of 5,000 CFU gave no information regarding the effect of antibiotic treatment because this inoculum apparently did not cause disease. In experiment 2, with an inoculum of 1.2×10^5 CFU, the disease that it caused was not severe enough to show the differential benefits of the different therapeutic regimens. In experiments 3 to 5, with large inocula, the benefits of the combined therapy over those of the other regimens was evident after 48 to 72 h (Table 2).

TABLE 1. Numbers of mice surviving after injection of different inoculum sizes of *V. vulnificus*

Time (h)	No. of surviving mice after injection of the following inoculum (CFU):					
	10^3	10^4	10^5	10^6	10^7	10^8
6	8	8	6	5	8	0
12	8	8	6	3	1	0
18	8	5	1	0	0	0
24	8	2	0	0	0	0
48	8	0	0	0	0	0

DISCUSSION

The experimental mouse model has previously been used for the study of *V. vulnificus* (3, 11, 22). To our knowledge, however, ours is the first to use this animal model as a tool for

TABLE 2. Effect of antibiotic therapy on survival of mice infected s.c. with *V. vulnificus*

Inoculum category and antibiotic dose	Inoculum size (no. of animals)	Time (h)	% mice in the following treatment groups surviving:				*P* value[a]
			Control	Cefotaxime	Minocycline	Combination	
Small inoculum standard dose	5×10^3 (9)	12	100	100	100	100	NS
		24	100	100	100	100	NS
		48	100	100	100	100	NS
		120	100	100	100	100	NS
Moderate inoculum, standard dose	1.2×10^5 (9)	12	78	100	100	100	NS
		24	22	100	100	100	0.0129
		48	22	100	100	100	0.0129
		120	22	100	100	100	0.0129
Large inoculum, standard dose	8×10^7 (9)	12	11	44	67	89	0.0074
		24	0	11	56	67	0.0032
		48	0	11	56	67	0.0032
		72	0	11	11	67[b]	0.004
		96	0	11	0	67[b]	0.001
		120	0	11	0	67[b]	0.001
Large inoculum, standard dose	1.2×10^8 (20)	6	0	75	80	75	<0.0001
		12	0	40	70	55	<0.0001
		24	0	20	45	45	<0.0009
		48	0	10	15	45[b]	0.0012
		72	0	0	5	40[b]	0.0001
		96	0	0	0	40[b]	<0.0001
		120	0	0	0	40[b]	<0.0001
Large inoculum five times the standard dose	6.6×10^7 (12)	6	25	75	58	92	0.0068
		12	0	33	58	92	<0.0001
		24	0	17	50	83	<0.0001
		48	0	8	42	83[b]	<0.0001
		72	0	8	42	83[b]	<0.0001
		96	0	8	33	83[b]	<0.0001
		120	0	8	33	83[b]	<0.0001

[a] The percentages of surviving mice in the treatment and control groups were compared by the Fisher-Freeman-Halton exact test. NS, not significant.
[b] A significant difference ($P < 0.05$) was found between combination therapy and minocycline therapy by a Scheffe-type post-hoc multiple comparison procedure.

evaluating the effects of combination antimicrobial therapy of *V. vulnificus* infection.

Tetracycline has generally been believed to be the drug of choice for the treatment of *Vibrio cholerae* as well as *V. vulnificus* infection. Bowdre et al. (3), for example, described an animal model in 1983. In their study, 12 of 12 mice given tetracycline survived, whereas 1 of 10 mice given cefotaxime survived. These data demonstrated the superiority of tetracycline over cefotaxime for the treatment of *V. vulnificus* infection in the mouse. In our hands, in the experiment with a large inoculum, mice treated with minocycline apparently lived longer than those treated with cefotaxime, but the observations were not substantiated by statistical analysis. On the other hand, our clinical experience has revealed that broad-spectrum cephalosporins are a good choice for the treatment of *V. vulnificus* infections (8, 9), although there are admittedly some confounding clinical factors, such as the severity of the disease at the time of the patient's arrival at the hospital and whether surgical debridement, which may influence the outcome, has been performed. In this study, the superiority of combined antibiotic treatment was most clearly demonstrated in the experiments with large inocula. Mice treated with either of the two antibiotics alone appeared grossly ill, while those treated with the combined antibiotic regimen remained healthy. The rationale behind the decision to test the efficacy of antibiotic therapy in animals with infections of different severities induced by various inoculum sizes lay in our concern that an antibiotic given singularly or in combination may be so overwhelmingly successful in suppressing the mild experimental infection in mice that the true synergistic effects of antibiotics used in combination, if operative, might be masked. On the contrary, if the infection is too severe, successful treatment might be too much to expect of the antibiotic under evaluation. In our study, the fact that minocycline and cefotaxime acted synergistically against an infection caused by a defined inoculum of *V. vulnificus* was unequivocally demonstrated. However, a question may arise, that is, that the pharmacokinetics of these antibiotics in mice given either separately or in combination may be quite different quantitatively from those in humans and that the relative lack of an effect of a single drug regimen that we have observed may be due to the use of inadequate therapeutic doses. Instead of determining the level of each antibiotic, we designed experiment 5, in which fivefold the recommended standard dose for humans was given to treat the infection. The results were reproducible. This result correlates well with our previous observations from in vitro studies (10). Our findings are especially valuable in the development of a therapeutic strategy for the treatment of severe wound infection caused by *V. vulnificus*. In treating a wound infection, one must be reminded that even without secondary bacteremia, a wound caused by *V. vulnificus* is always accompanied by severe local swelling, necrosis, and vessel occlusion and thrombosis, which might compromise the blood supply. These changes make it difficult to attain an adequate antibiotic level in tissue. Combination therapy might have the greatest effect in

patients with this condition, although early surgical debridement can never be omitted (5, 8).

In conclusion, this study demonstrated the superiority of combining cefotaxime and minocycline for the treatment of severe *V. vulnificus* infection in the mouse. While our unpublished clinical data are also sufficient to convince us of the clear advantage of the same chemotherapeutic strategy for the treatment of humans, whether it may be foiled by the existence of concurrent liver cirrhosis or other immunocompromising conditions in the patient is an important question that has yet to be answered.

ACKNOWLEDGMENTS

This work was partly supported by grants (NSC86-2314-B006-056 and NSC87-2314-B006-042) from the National Science Council and was partly supported by grants (NCKUH 85-001 and NCKUH 86-002) from National Cheng Kung University Hospital, Tainan, Taiwan, Republic of China.

REFERENCES

1. **Blake, P. A., M. H. Merson, R. E. Weaver, D. G. Hollis, and P. C. Heublein.** 1979. Disease caused by a marine *Vibrio*: clinical characteristics and epidemiology. N. Engl. J. Med. **300:**1–5.
2. **Bonner, J. R., A. S. Coker, C. R. Berryman, and H. M. Pollock.** 1983. Spectrum of vibrio infections in a Gulf Coast community. Ann. Intern. Med. **99:**464–469.
3. **Bowdre, J. H., J. H. Hull, and D. M. Cocchetto.** 1983. Antibiotic efficacy against *Vibrio vulnificus* in the mouse: superiority of tetracycline. J. Pharmacol. Exp. Ther. **225:**595–598.
4. **Chang, J. J., I. S. Sheen, S. M. Peng, P. C. Chen, C. S. Wu, and H. S. Leu.** 1994. *Vibrio vulnificus* infection: report of 8 cases and review of cases in Taiwan. Chang. Gung. Med. J. **17:**339–345.
5. **Chuang, Y. C., C. Young, and C. W. Chen.** 1989. *Vibrio vulnificus* infection. Scand. J. Infect. Dis. **21:**721–726.
6. **Chuang, Y. C., and C. D. Young.** 1989. *Vibrio vulnificus* infection: clinical experience with 7 cases, abstr., p. 114–115. *In* Proceedings of the Formosan Medical Association Annual Meeting 1989. Formosan Medical Association, Taipei, Taiwan.
7. **Chuang, Y. C.** 1990. *Vibrio vulnificus* infection in Taiwan, p. 108. *In* Proceedings and abstracts of the 2nd Western Pacific Congress on Infectious Diseases and Chemotherapy. Infectious Diseases Association of Thailand and Western Pacific Society of Chemotherapy, Jomtien-Pattaya, Thailand.
8. **Chuang, Y. C., C. Y. Yuan, C. Y. Liu, C. K. Lan, and A. H. M. Huang.** 1992. *Vibrio vulnificus* infection in Taiwan: report of 28 cases and review of clinical manifestations and treatment. Clin. Infect. Dis. **15:**271–276.
9. **Chuang, Y. C.** 1992. Clin. Infect. Dis. **15:**1072. (Letter.)
10. **Chuang, Y. C., J. W. Liu, W. C. Ko, K. Y. Lin, J. J. Wu, and K. Y. Huang.** 1997. In vitro synergism between cefotaxime and minocycline against *Vibrio vulnificus*. Antimicrob. Agents Chemother. **41:**2214–2217.
11. **Chuang, Y. C., H. M. Sheu, W. C. Ko, T. M. Chang, M. C. Chang, and K. Y. Huang.** 1997. Mouse skin damage caused by a recombinant extracellular metalloprotease from *Vibrio vulnificus* and by *V. vulnificus* infection. J. Formos. Med. Assoc. **96:**677–684.
12. **Fang, F. C.** 1992. Use of tetracycline for treatment of *Vibrio vulnificus* infection. Clin. Infect. Dis. **15:**1071–1072. (Letter.)
13. **Freeman, G. H., and J. H. Halton.** 1951. Note on an exact treatment of contingency, goodness of fit and other problems of significance. Biometrika **38:**141–149.
14. **Hsueh, P. R., J. C. Chang, S. C. Chang, S. W. Ho, and W. C. Hsieh.** 1995. In vitro antimicrobial susceptibility of *Vibrio vulnificus* isolated in Taiwan. Eur. J. Clin. Microbiol. Infect. Dis. **14:**151–153. (Letter.)
15. **Kelly, M. T., and D. M. Avery.** 1980. Lactose-positive *Vibrio* in seawater: a cause of pneumonia and septicemia in a drowning victim. J. Clin. Microbiol. **11:**278–280.
16. **Kelly, M. T., and W. F. McCormick.** 1981. Acute bacterial myositis caused by *Vibrio vulnificus*. JAMA **246:**72–73.
17. **Klontz, K. C., S. Lieb, M. Schreiber, H. T. Janowski, L. M. Baldy, and R. A. Gunn.** 1988. Syndromes of *Vibrio vulnificus* infections: clinical and epidemiologic features in Florida cases, 1981–1987. Ann. Intern. Med. **109:**318–323.
18. **Marascuilo, L. A., and M. McSweeney.** 1977. Nonparametric and distribution-free methods for the social science. Brooks/Cole Company, Monterey, Calif.
19. **Morris, J. G., Jr., and R. E. Black.** 1985. Cholera and other vibrioses in the United States. N. Engl. J. Med. **312:**343–350.
20. **Morris, J. G., Jr., and J. Tenny.** 1985. Antibiotic therapy for *Vibrio vulnificus* infection. JAMA **252:**1221–1222. (Letter.)
21. **Park, S. D., H. S. Shon, and N. J. Joh.** 1991. *Vibrio vulnificus* septicemia in Korea: clinical and epidemiologic findings in seventy patients. J. Am. Acad. Dermatol. **24:**397–403.
22. **Poole, M. D., and J. D. Oliver.** 1978. Experimental pathogenicity and mortality in ligated ileal loop studies of the newly reported halophilic lactose-positive *Vibrio* sp. Infect. Immun. **20:**126–129.
23. **Sanford, J. P., D. N. Gilbert, R. C. Moellering, Jr., and M. A. Sande.** 1997. Selection of initial empirical antibacterial therapy on clinical grounds, p. 2–44. *In* J. P. Sanford, D. N. Gilbert, R. C. Moellering, Jr., and M. A. Sande (ed.), The Sanford guide to antimicrobial therapy, 27th ed. Antimicrobial Therapy Inc., Dallas, Tex.
24. **Yuan, C. Y., C. C. Yuan, D. C. Wei, and A. M. Lee.** 1987. Septicemia and gangrenous change of the legs caused by marine vibrio, *Vibrio vulnificus*—report of a case. J. Formos. Med. Assoc. **86:**448–451.