ANTIMICROBIAL AGENTS AND CHEMOTHERAPY. Oct. 1996. p. 2296-2299
0066-4804/96/$04.00+0

Vol. 40. No. 10

Bactericidal Action of Ofloxacin, Sulbactam-Ampicillin, Rifampin, and Isoniazid on Logarithmic- and Stationary-Phase Cultures of *Mycobacterium tuberculosis*

DANIEL HERBERT,[1] C. N. PARAMASIVAN,[1] P. VENKATESAN,[1] G. KUBENDIRAN,[1] R. PRABHAKAR,[1] AND D. A. MITCHISON[2]*

Tuberculosis Research Centre, Indian Council of Medical Research, Madras 600 031, India, [1] and St. George's Hospital Medical School, London SW17 0RE, United Kingdom

Received 17 May 1996/Returned for modification 9 July 1996/Accepted 7 August 1996

The bactericidal actions of ofloxacin and sulbactam-ampicillin, alone and in combination with rifampin and isoniazid, on exponential-phase and stationary-phase cultures of a drug-susceptible isolate of *Mycobacterium tuberculosis* were studied in vitro. In exponential-phase cultures, all drugs were bactericidal. with the higher concentrations of ofloxacin (5 μg/ml) and sulbactam-ampicillin (15 μg of ampicillin per ml) being as bactericidal as 1 μg of isoniazid per ml or 1 μg of rifampin per ml. In two-drug combinations. both drugs increased the levels of activity of isoniazid and rifampin and were almost as bactericidal as isoniazid-rifampin; they also appeared to increase the level of activity of isoniazid-rifampin in three-drug combinations. In contrast. ofloxacin and sulbactam-ampicillin had little bactericidal activity against stationary-phase cultures and were less active than isoniazid or rifampin alone. Furthermore. in two-drug or three-drug combinations, they did not increase the level of activity of isoniazid, rifampin, or isoniazid-rifampin. These findings suggest that ofloxacin and sulbactam-ampicillin are likely to be most useful in the early stages of treatment and in preventing the emergencc of resistance to other drugs hut are unlikely to be effective as sterilizing drugs helping to kill persisting lesional bacilli.

The activities of antituberculosis drugs have been postulated to occur in two phases (12). In the first phase the predominant action is the killing of actively growing *Mycobacterium tuberculosis.* This can be measured in patients with pulmonary tuberculosis by the early bactericidal activity (EBA) of the drug. which is the fall in the numbers of viable tubercle bacilli in the sputum during the first few days of treatment (9). In the second sterilizing phase, which may also start early in treatment but continues longer than the first phase, the drug kills semidormant organisms which constitute the remaining bacilli. Sterilizing efficacy is measured as the relapse rate after the end of chemotherapy-and as the proportion of sputum cultures that are negative at 3 months (13). Individual drugs differ greatly in their relative activities during the early bactericidal and sterilizing phases (11). For example. isonkid has a high level of EBA but only limited sterilizing activity. whereas pyrazinamide has a negligible EBA but considerable sterilizing activity. Both types of activity in the combination of drugs are necessary for effective chemotherapy. A high level of EBA seems to be associated with the ability to prevent the emergence of drug resistance, while a high level of sterilizing activity shortens the duration of chemotherapy. It has been suggested that in vitro measurement of bactericidal activity against exponential-phase *M. tuberculosis* may reflect EBA whereas bactericidal activity against stationary-phase bacilli may reflect the sterilizing activity of the drug (12). We have explored the activities against exponential- and stationary-phase cultures of the 5-fluoroquinolone ofloxacin (18-20) and of the ampicillin-sulbactam combination (1, 5, 16, 17), in which sulbactam inhibits the bacterial penicillinase, because they are of value in the treatment of

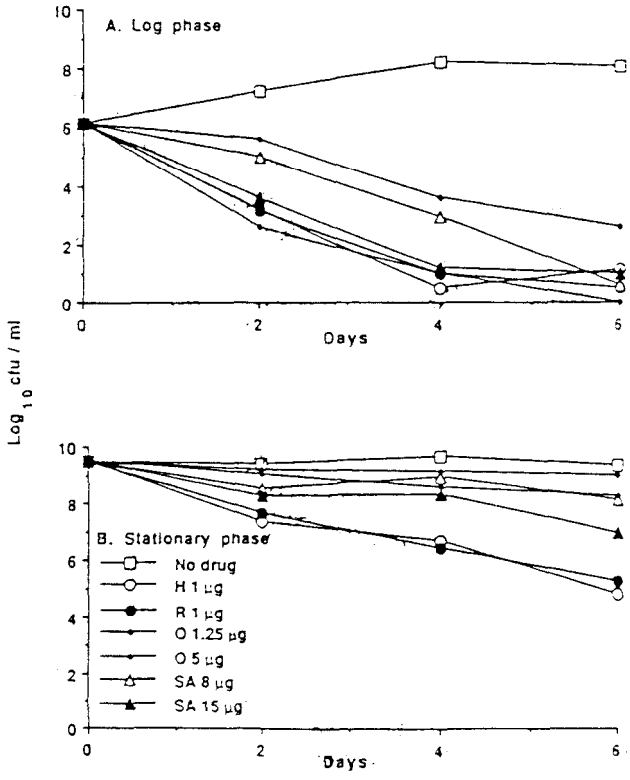

FIG. 1. Bactericidal activities against log-phase (A) and stationary-phase (B) cultures of *M. tuberculosis* of isoniazid at 1 μg/ml (H 1 μg), rifampin at 1 μg/ml (R 1 μg), ofloxacin at 1.25 μg/ml (O 1.25 μg), ofloxacin at 5 μg/ml (O 5 μg), sulbactam-ampicillin at 8 μg/ml (SA 8 μg), and sulbactam-ampicillin at 16 μg/ml (SA 16 μg).

* Corresponding author. Mailing address: Department of Medical Microbiology, St. George's Hospital Medical School, London SW17 ORE. United Kingdom. Phone: 0181-725 5704. Fax: 0181-682 1320. Electronic mail address: dmitchis@sghms.ac.uk.

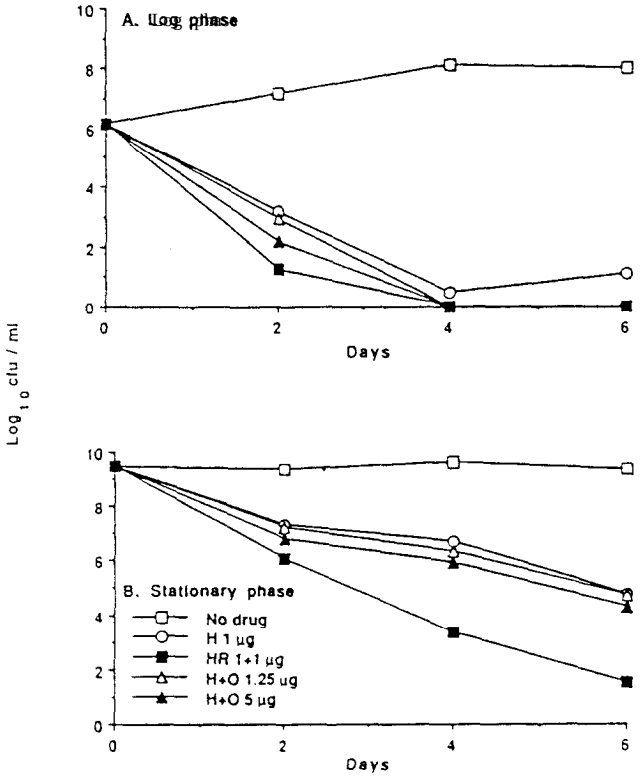

FIG. 2. Bactericidal activities against log-phase (A) and stationary-phase (B) cultures of *M. tuberculosis* of isoniazid alone (H 1 µg) and with rifampin (HR 1+1 µg) or ofloxacin (H+O 1.25 µg and H+O 5 µg) at the indicated drug concentrations.

patients with multidrug-resistant strains. We have compared ofloxacin and ampicillin-sulbactam with isoniazid and rifampin, alone and in combination. Bactericidal activity against stationary-phase organisms has only rarely been measured. and there may be several ways in which the stationary state could be induced by. for instance. a lack of nutrients (7) or by anaerobiosis, low temperature, or suboptimal pH (3). Several of these conditions increase production of mRNA from the static y-phase stress response sigma factor sigF of *M. tuberculosis* (2). We have chosen cultures made stationary by prolonged incubation in tightly sealed screw-cap bottles, which probably limits growth because of a lack of oxygen and promotes an adaptation to an anaerobic environment (21); this condition may reflect the limitation of growth in human lesions, particularly those which do not have direct bronchial access to air.

MATERIALS AND METHODS

Chemotherapeutic drugs. Stock solutions were prepared from pure powders of ofloxacin, sulbactam, ampicillin, rifampin, and isoniazid. They were sterilized by filtration through 0.22-µm-pore-size membrane filters and stored at 4°C.

Cultures of *M. tuberculosis*. The experiments were done with a fresh, drug-susceptible isolate (TS51476) obtained from a patient with no history of previous treatment, as well as with strain H37Rv. Log-phase and stationary-phase cultures of the strains were prepared as follows (4). The organisms were grown in 10 ml of 7H9 Tween- 80-albumin medium (Difco Laboratories, Detroit. Mich.) for 7 days at 37°C. The total number of bacilli per milliliter in this culture was determined in a Thoma chamber, and an appropriate volume was inoculated into two flasks. each containing 500 ml of fresh 7H9 medium, to give 10^5 bacilli per ml. One flask was incubated at 37°C for 3 days (exponential-phase culture). The other flask was incubated at 37°C for 4 weeks (stationary-phase culture), during which time growth continued mechanically undisturbed under a 5-cm layer of medium.

Bactericidal action of the drugs. The exponential-phase and stationary-phase cultures were distributed without dilution or concentration by centrifugation into duplicate 10-ml aliquots in 28-ml screw-cap McCartney bottles (day 0). Drugs were added about 1 h later as follows: isoniazid, rifampin, ofloxacin, sulbactam-ampicillin, isoniazid-ofloxacin, isoniazid-sulbactam-ampicillin, rifampin-ofloxacin, rifampin-sulbactam-ampicillin, isoniazid-rifampin, isoniazid-rifampin-ofloxacin, and isoniazid-rifampin-sulbactam-ampicillin. Ofloxacin was added at two final concentrations of 1.25 µg/ml, just above the MlC (6), and 5 µg/ml, the peak concentration attainable with the extent dosage for human disease. Sulbactam-ampicillin was added at two final concentrations of ampicillin. 8 and 15 µg/ml. with sulbactam and ampicillin in a 1:2 ratio. Rifampin and isoniazid were added to give a final concentration of 1 µg/ml each. Drug-free control cultures were included. In the cultures with drugs, additional drugs were added on day 3 (50% of the amounts of sulbactam-ampicillin, rifampin, and isoniazid and 10% of the amount of ofloxacin added on day 0) to increase the concentrations by 150% of the losses during the 3 days, as estimated from standardization expcr-iments (4). Counts of CFU were taken from serial 10-fold dilutions of the cultures on duplicate plates of selective H11 medium containing polymyxin B (200 U/ml), amphotericin B (10 µg/ml, carbenicillin (100 µg/ml), and trimcthoprim (10 µg/ml) (4). A CFU count was taken from flasks at day 0 and from all the cultures at days 2, 4, and 6. The plates were incubated in polythene bags at 37°C, and colonies were counted after 2 and 4 weeks of incubation.

RESULTS

The results of the two experiments, one on the exponential-phase culture and the other on the stationary-phase culture. could not be presented as single graphs because of the complexity of the data. Different comparisons within the same experiment are therefore presented in successive figures. The CFU counts from the exponential- and stationary-phase cultures of *M. tuberculosis* exposed to the single drugs are shown in Fig. 1. As expected, there was continued growth of the drug-tree exponential-phase culture hut no growth of the stationary-phase culture. In the exponential-phase cultures, rifampin, isoniazid and ofloxacin and sulbactam-ampicillin at the higher concentrations (5 and 15 µg/ml, respectively) all had similar bactericidal activities whereas ofloxacin and sulbactam-

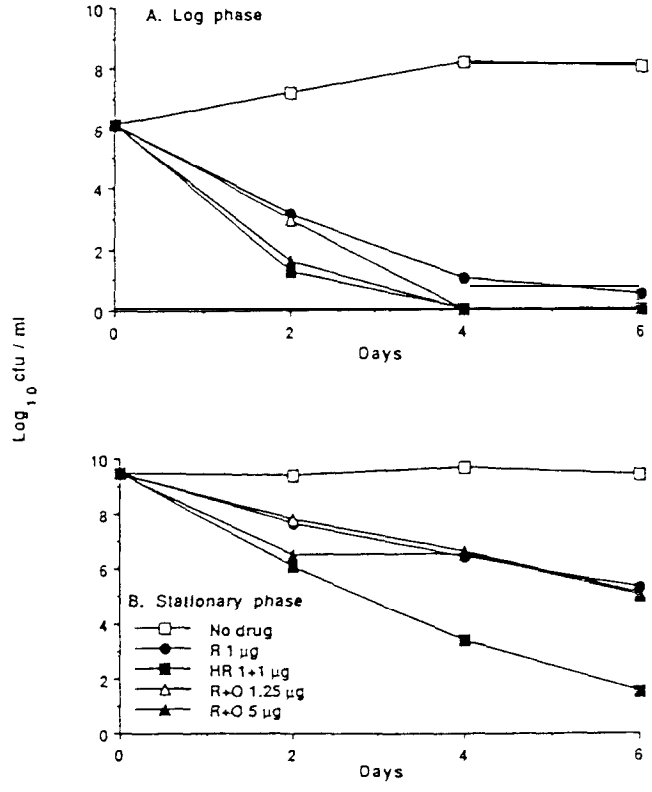

FIG. 3. Bactcricidal activities against log-phase (A) and stationary-phase (B) cultures of *M. tuberculosis* of rifampin alone (R 1 µg) and with isoniazid (HR 1+1 µg) or ofloxacin (R+O 1.25 µg and R+O 5 µg) at the indicated drug concentrations.

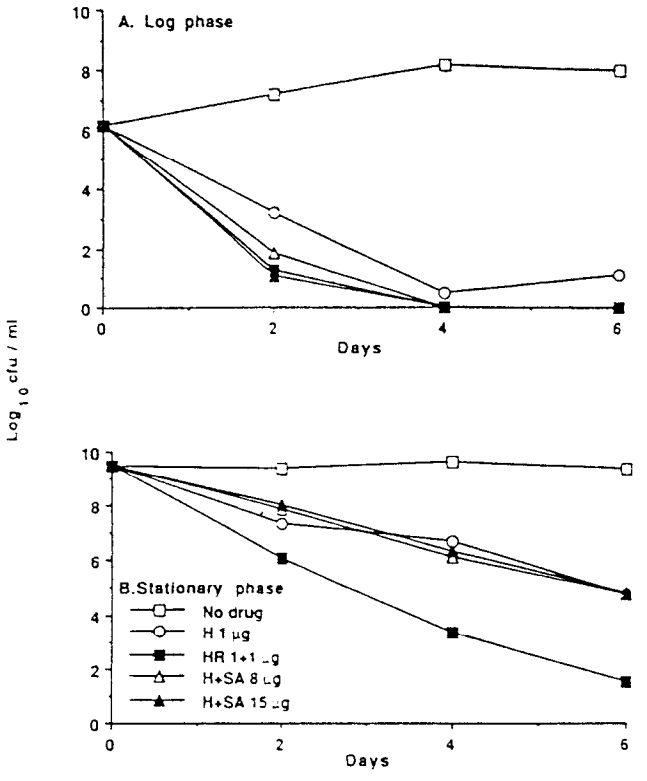

FIG. 4. Bactericidal activities against log-phase (A) and stationary-phase (B) cultures of *M. tuberculosis* of isoniazid alone (H 1 µg) and with rifampin (HR 1+1 µg) or sulbactam-ampicillin (H+SA 8 µg and H+SA 15 µg) at the indicated drug concentrations.

ampicillin at the lower concentrations (1.25 and 8 µg/ml, respectively) showed lesser bactericidal activities. On stationary-phase cultures, however. rifampin and isoniazid retained moderate levels of bactericidal activity while ofloxacin and sulbactam-ampicillin, even at their higher concentrations, had much less activity.

The effects of adding ofloxacin and sulbactam-ampicillin to isoniazid, rifampin, and isonizid-rifampin are shown in Fig. 2 to 6. In each figure illustratin g additions to isoniazid (Fig. 2 and 4), the same curve for isoniazid alone is shown for comparison. Similarly, the same reference curve for rifampin alone is shown when ofloxacin and sulbactam-ampicillin were added to rifampin (Fig. 3 and 5) and the same reference curve for isoniazid-rifampin, which was more bactericidal than isoniazid or rifampin alone, is shown in Fig. 2 to 6. In exponential-phase cultures, when ofloxacin was added to isoniazid or rifampin, each of the combinations with the higher ofloxacin concentration (5 µg/ml) was nearly as bactericidal as isoniazid-rifampin (Fig. 2A and 3A) while the combinations with the lower ofloxacin concentration were somewhat less bactericidal. In contrast, in stationary-phase cultures, the additions of ofloxacin had hardly any effect on the activity of isoniazid or rifampin and the combinations were much less active than isoniazid-rifampin (Fig. 2B and 3B). Similar results were obtained when sulbactam-ampicillin was added to isoniazid (Fig. 4) or rifampin (Fig. 5). In exponential-phase cultures, sulbactam-ampicillin increased the level of activity of isoniazid or rifampin, almost to the same extent as isoniazid-rifampin, but in stationary-phase cultures, neither concentration of sulbactam-ampicillin appreciably altered the activity of either isoniazid or rifampin and the combinations were much less active than isoniazid-rifampin. Thus, the ability of ofloxacin and sulbactam-ampicillin, particularly at their higher concentrations, to increase the bactericidal activity of isoniazid or rifampin was lost as the culture changed from exponential phase to stationary phase. When ofloxacin or sulbactam-ampicillin was added to isoniazid-rifampin (Fig. 6), there was probably an increase in the level of bactericidal activity against exponential-phase organisms evident in the 2-day CFU counts with all three-drug combinations but there was no corresponding increase in the stationary-phase culture. In summary, therefore, synergistic bactericidal effects between ofloxacin or sulbactam-ampicillin and isoniazid, rifampin, or isoniazid-rifampin were evident in exponential-phase cultures but were absent in the stationary phase cultures.

In the experiments with strain H37Rv, ofloxacin and sulbactam-ampicillin at their higher concentrations of 5 and 15 µg/ml, respectively, were highly bactericidal but ofloxacin at 1.25 µg/ml had a lower level of activity and sulbactam-ampicillin at 8 µg/ml had even less activity. Unfortunately, there was so little bactericidal activity of any drug or drug combination on stationary-phase cultures that it was impossible to measure their relative effects.

DISCUSSION

Ofloxacin is well established in reserve drug treatment of tuberculosis, as it is moderately effective and has little toxicity even at the high daily dose level of 800 mg (8). There is however, little information on the value of sulbactam-ampicillin, though results in the treatment of leprosy (15) and with a similar combination of amoxicillin and clavulanic acid in two patients with multidrug-resistant strains are encouraging (14). Even though plasma ampicillin concentrations as high as 8 to

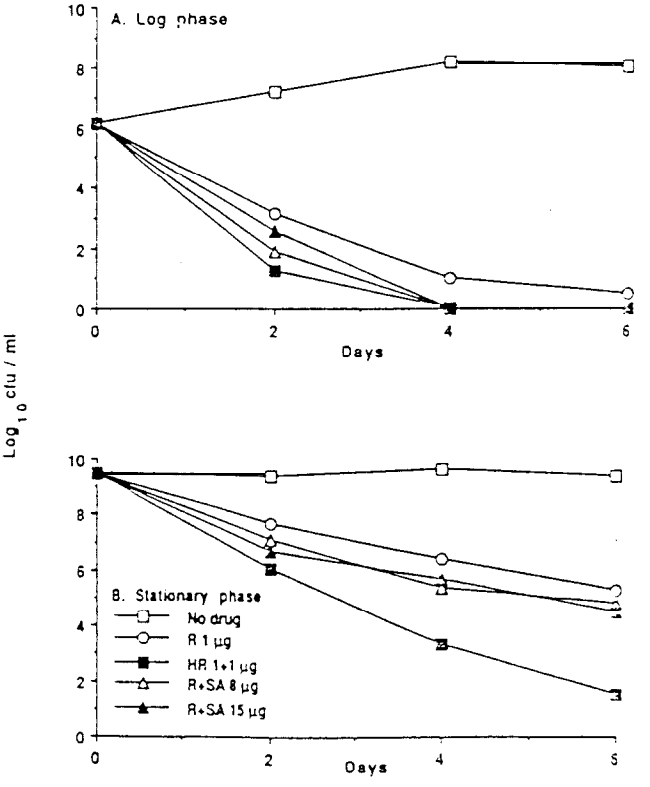

FIG. 5. Bactericidal activities against log-phase (A) and stationary-phase (B) cultures of *M. tuberculosis* of rifampin alone (R 1 µg) and with isoniazid (HR 1+1 µg) or sulbactam-ampicillin (R+SA 8 µg and R+SA 15 µg) at the indicated drug concentrations.

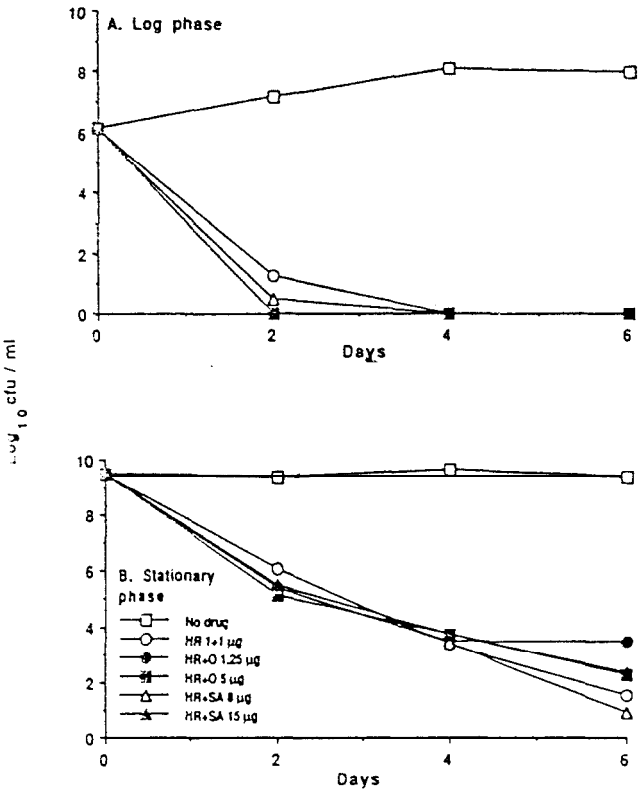

FIG. 6. Bactericidal activities against log-phase (A) and stationary-phase (B) cultures of *M. tuberculosis* of isoniazid-rifampin alone (HR 1+1 µg) or with ofloxacin (HR+O 1.25 µg and HR+O 5 µg) or sulbactam-ampicillin (HR+SA 8 µg and HR+SA 15 µg) at the indicated drug concentrations.

15 µg/ml may be difficult to maintain, the use of sulbactam-ampicillin needs further exploration.

The results of the present study suggest that both drugs would be particularly useful in the first few days of treatment of pulmonary tuberculosis, when the majority of the bacterial population is actively multiplying in cavity walls well supplied with air and when the risk of selection of mutants resistant to accompaning drugs is greatest. For the same reason, they would also be valuable in killing bacilli that grow actively after a bacteriological relapse that happens during or after the end of treatment. In view of their considerable bactericidal activity against the exponential-phase culture. they would be expected to have fairly high bactericidal activity at the start of treatment. No estimates have been published of the EBA of either drug, but the EBA of ofloxacin is currently being investigated in a multicenter study supported by the World Health Organization. On the other hand. the low level of bactericidal activity on stationary-phase cultures suggests that, though they would-still continue to inhibit growth, neither drug would be effective in shortening the duration of treatment. Thus. if the drug combination does not contain another sterilizing drug, treatment should be continued for at least 12 months, as was the custom before the advent of the effective sterilizing drugs rifampin and pyrazinamide. There is no direct evidence of the sterilizing activity of ofloxacin in human disease. However, a study has been done of experimentally induced murine tuberculosis with sparfloxacin, a quinolone with lower MICs against *M. tuberculosis* and favorable pharmacodynamics, which showed it to have a high level of bactericidal activity when it was given alone but which failed to find any increase in the level of sterilizing activity when it was added to a regimen of isoniazid, rifampin, and pyrazinamide (10).

In conclusion, routine estimation of the bactericidal activities of drugs against stationary- or near-stationan-phase cultures of *M. tuberculosis* is desirable in the preclinical phase of drug development as an indication of sterilizing activity. More work is, however, necessary to establish this method of assessment by comparing its results with estimates of sterilizing activities of drugs against experimentally induced murine tuberculosis and, in patients with pulmonary tuberculosis, by comparison with relapse rates after chemotherapy and the proportion of patients with early sputum conversion (13).

ACKNOWLEDGEMENTS

Ofloxacin was kindly provided by Dominion Chemical Industries Ltd., Bangalore, India, far Hoechst, Bombay, India; sulbactam and ampicillin were provided by Unichem Laboratories Ltd., Bombay, India; and isoniazid was provided by Bayers, Levcrkusen, Germany; rifampin was purchased from Sigma.

REFERENCES

1. Bush, K. 1988. Beta-lactamase inhibitors from laboratory to clinic. Clin. Microbiol. Rev. **1:** 109-13.
2. **DeMaio, J., Y. Zhang, C. Ko, D. G. Young, and W. R. Bishai.** 1996. A stationary-phase stress-response sigma factor from *Mycobacterium tuberculosis*. Proc. Natl. Acad. Sci. USA 93:2790–2794.
3. **Dickinson, J. M., and D. A. Mitchison.** 1981. Experimental models to explain the high sterilizing activity of rifampin in the chemotherapy of tuberculosis. Am. Rev. Respir. Dis. 123:367–371.
4. **Dickinson, J. M., and D. A. Mitchison.** 1990. In vitro activities against mycobacteria of two long acting rifamycins, FCE 22807 and CGP 40469A (SPA-S-565). Tubercle 71:109–115.
5. **Foulds, G.** 1986. Pharmacokinetics of sulbactam/ampicillin in humans: a review. Rev. Infect. Dis. 8(Suppl. 5):S503–S511.
6. **Heifets, L. B., and P. J. Lindholm-Levy.** 1987. Bacteriostatic and bactericidal activity of ciprofloxacin and ofloxacin against *Mycobacterium tuberculosis* and *Mycobacterium avium* complex. Tubercle 68:267–276.
7. **Hobby, G. L., and T. F. Lenert.** 1957. The in vitro action of antituberculous agents against multiplying and non-multiplying microbial cells. Am. Rev. Tuberc. Pulm. Dis. 6:1031–1048.
8. Hong Kong Chest Service/British Medical Research Council, 1992. A controlled study of rifabutin and an uncontrolled study of ofloxacin in the retreatment of patients with pulmonary tuberculosis resistant to isoniazid, streptomycin and rifampicin. Tubercle Lung Dis. **73:** 59-67.
9. Jindani, A., V. R Aber, E. A. Edwards. and D. A. Mitchison, 1980. The early bactericidal activity of drugs in patients with pulmonary tuberculosis. Am. Rev. Respir. Dis. **121:** 939-949.
10. Lalande, V., C. Truffot-Pernot, A. P. Moulin, J. Grossct, and B. Ji. 1993. Powerful bactericidal activity of sparfloxacin (AT-4140) against *Mycobacterium tuberculosis* in mice. Annmicrob. Agents Chemothcr. **37:** 407-13.
11. Mitchison, D. A. 1985. The action of antituberculosis drugs in short course chemotherapy. Tubcrcle **66:** 219-225.
12. Mitchison, D. A. 1992. The Garrod lecture. Understanding the chemotherapy of tuberculosis-current problem. J. Antimicrob. Chemother. **29:** 477-493.
13. Mitchison, D. A. 1993. Assessment of new sterilizing drug for treating pulmonary tuberculous by culture at 2 months. Am. Rev. Rcspir. Dis. **147:** 1062-1063
14. Sadler, J. P., J. Berger, J. A. Nord, R. Cotsky, and Ml. Saxena. 1991. Amoxicilin-clavulanic acid for treating drug-resistant *Mycobacterium tuberculosis.* Chest **99:** 1025-1016.
15. Prabhakaran, K., E. B. Harris, B. Randhawa, and R C. Hastings. 1992. Reversal of drug resistance in *Mycobacterium leprae* by ampicillin/sulbactam. Microbios **72:** 137-142.
16. Ripa, S., L. Ferrante, and M. Prenna. 1990. Pharmacokinetics of sulbactam/ampicillin in humans after intravenous and intramuscular injection. Chemotherapy (Basel) **36:** 185-192.
17. Sorg, T. B, and M. H. Cynamon. 1987. Comparison of four beta-lactamase inhibitors in combination with ampicillin against *Mycobacterium tuberculosis.* J. Antimicrob. Chemothcr. **19:** 59-64.
18. Tsukamura, M. 1985. In vitro antituberculosis activity of a new antibacterial substance, Ofloxacin (DL 8280). Am. Rev. Respir. Dis. **131:** 348-351.
19. Tsukamura, M. 1985. Antituberculosis activity of ofloxacin (DL 8280) on cxperimental tuberculosis in mice. Am. Rev. Rcspir. Dis. **135:** 915.
20. Venkataraman, P, C. N. Paramasivan, and R Prabhakar. 1994 In vitro activity of ciprofloxacin and ofloxacin against South Indian isolates of *Mycobacterium tuberculosis.* Indian J. Tuberc. **41:** 87-90.
21. Waync, L G. 1994. Dormancy of *Mycobacterium tuberculosis* and latency of disease. Eur. J. Clin. Microbiol. Infect. Dis. **13:** 908-914.