ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, June 1987, p. 930–934
0066-4804/87/060930-05$02.00/0

Vol. 31, No. 6

Quantitative Effect of Granulocytes on Antibiotic Treatment of Experimental Staphylococcal Infection

JAAP J. HOOGETERP, HERMAN MATTIE,* ANNEMIEK M. KRUL, AND RALPH VAN FURTH

Department of Infectious Diseases, University Hospital, 2300 RC Leiden, The Netherlands

Received 2 September 1986/Accepted 23 March 1987

The quantitative relation between granulocytopenia and antibiotic treatment was established for a short-term *Staphylococcus aureus* infection in the thighs of mice, using rifampin, benzylpenicillin, and erythromycin. Granulocytopenia was induced by total-body irradiation; the number of granulocytes decreased gradually during the first 5 days after irradiation to 10% of the number in normal mice. Experimental infections were established on each of the 5 days after irradiation. In animals not treated with antibiotics, the number of granulocytes in peripheral blood and the number of CFU at the site of infection exhibited a strong negative correlation. The influence of granulocytes on the effect of antibiotics on the number of CFU differed for the three antibiotics. For erythromycin the slope of the dose-effect relation was rather flat, but a decrease in the number of granulocytes caused a significant, nearly parallel, shift in the dose-effect relation, resulting in an increase in the number of CFU. For benzylpenicillin the slope of the dose-effect relation for normal mice was also flat, but as the number of granulocytes decreased the slope became significantly steeper, resulting in a diminishing influence of granulocytes at higher dosages. For rifampin the slope of the dose-effect relation, which was already steep for nonirradiated animals, increased significantly. Here too the effect of granulocytes decreased as the dose increased.

Natural resistance to infections is the result of many factors, including intact skin and mucous membranes, a sufficient number of phagocytic cells, an optimal concentration of immunoglobulins, and a normal complement system (6). Irradiation affects the proliferation of the precursors of phagocytic cells in bone marrow, leading to granulocytopenia and monocytopenia which decrease resistance to infections (3, 5, 13, 17).

In granulocytopenic patients the outcome of antibiotic treatment of bacterial infections is often disappointing, which indicates that phagocytic cells are needed for successful antibiotic treatment. However, the relation between the effect of antibiotics and that of phagocytic cells is still poorly understood. Animal models can be used to quantitate the effect of each of these factors alone as well as their mutual interaction on bacteria in an experimental infection (8, 12, 15). In previous studies from our laboratory, the effect of antibiotics that are effective against gram-negative microorganisms was studied in an experimental thigh infection in mice (8, 11, 14–16). The present study was designed to obtain more detailed information on the influence of the number of granulocytes on the dosage of various antibiotics against *Staphylococcus aureus*, using the same experimental infection in mice.

MATERIALS AND METHODS

Antibiotics. Erythromycin (base, 98.4% activity) was obtained from Abbott BV, Amstelveen, The Netherlands; benzylpenicillin (potassium salt, 99.8% activity) was from Gist-Brocades NV, Delft, The Netherlands; rifampin (98.9% activity) was obtained from Lepetit, Rotterdam, The Netherlands. Standard solutions of erythromycin and benzylpenicillin were prepared by dissolving the drugs in phosphate-buffered saline (PBS) (pH 7.5). A standard solution of rifampin was obtained by dissolving the antibiotic in 100 μl of dimethyl sulfoxide (Serva, Heidelberg, Federal Republic of Germany) and then diluting the solution with PBS.

Microorganisms. *S. aureus* 42D was stored as a suspension of about 10^9 CFU/ml in brain heart infusion (Oxoid Ltd., Basingstoke, U.K.) in vials of 1.5 ml at −70°C. Before each experiment a vial was quickly thawed at 37°C. The MICs of erythromycin, benzylpenicillin, and rifampin for this microorganism were 0.25, 0.032, and 0.008 μg/ml, respectively; the MBCs of benzylpenicillin and rifampin were 0.032 and 0.016 μg/ml, respectively. The microorganism used was serum resistant.

Animals. Male, specific-pathogen-free Swiss mice weighing 20 to 25 g were obtained from the Central Institute for the Breeding of Laboratory Animals, Zeist, The Netherlands.

In vitro growth curves in murine serum. To determine whether humoral factors affect the growth of the microorganism or interact with the antibiotics, in vitro growth studies were performed in the presence of serum from normal and irradiated mice. About 10^6 CFU/ml were incubated in serum diluted to 90% with PBS. Bacteria were also cultured in the presence of erythromycin at 1 μg/ml, benzylpenicillin at 0.032 μg/ml, or rifampin at 0.032 μg/ml; at these total concentrations the concentrations not bound to murine protein were approximately equal to the respective MICs (unpublished results). At given times, 50-μl samples of the cultures were taken and serial dilutions with PBS were made. Appropriate dilutions were plated on DST agar (Oxoid Ltd.), and colonies were counted after incubation for 18 h at 37°C.

Irradiation of mice. Mice were placed in Plexiglas containers (15 by 15 by 10 cm) divided into two chambers of equal size, each chamber holding 10 mice. The animals were irradiated forward and backward, receiving a dose of 6 Gy delivered by a 5-MV linear accelerator (Philips SL 75-5; Philips, Eindhoven, The Netherlands).

Quantitation of blood leukocytes. A blood sample (50 μl) taken from the retro-orbital plexus of each mouse was collected in plastic vials containing 10 μl (400 U/ml) of

* Corresponding author.

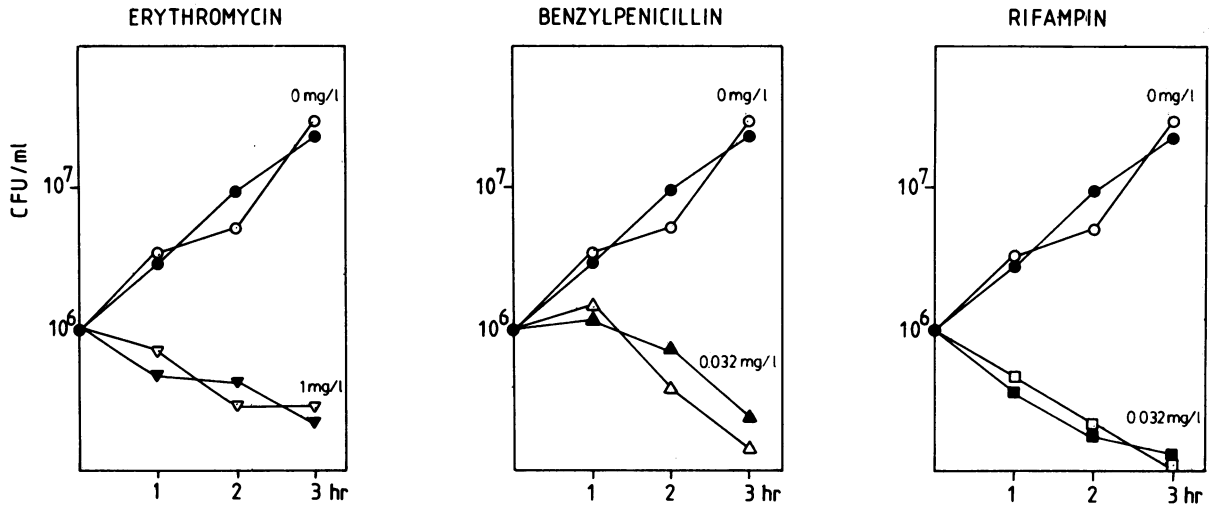

FIG. 1. Number of *S. aureus* CFU in vitro in the absence or presence of antibiotics in the serum of irradiated (open symbols) and normal (solid symbols) mice.

heparin in saline (7). Total leukocyte counts were obtained in triplicate with a Coulter counter (model FN; Coulter Electronics Ltd., Luton, U.K.). The granulocytes, lymphocytes, and monocytes in a Giemsa-stained blood smear were counted to a total of 300 cells per blood smear. The numbers of granulocytes, monocytes, and lymphocytes per liter of blood were calculated from the total leukocyte count and the differential counts. Leukocytes were counted immediately before infection was induced.

Experimental design. On days 0 through 5 after irradiation, mice were infected by injecting into the thigh muscle approximately 10^6 CFU of *S. aureus* which had been stored at −70°C and rapidly thawed. After the bacteria had proliferated in the thigh muscle for 1 h, antibiotics were administered subcutaneously in various dosages. Control animals received saline. Dosages were chosen such that the lowest dose of each antibiotic gave a substantial difference in CFU counts with respect to controls while the highest dose yielded a result that was slightly lower than the maximal effect. At 4h after administration of the antibiotic, the animals were killed by cervical dislocation, and the infected thigh muscles were excised and homogenized for 1 min at 0°C in 5.0 ml of PBS in Potter-Elvejhem tubes. Serial dilutions of the homogenate were made, and the appropriate dilutions were plated out on DST agar as described above. In a separate experiment, antibiotic concentrations were determined by microbiological assay (10, 11). The detection level of the assay was 0.06 μg/ml for all three antibiotics.

RESULTS

In vitro growth in murine serum. *S. aureus* incubated in the presence of 90% normal murine serum or serum from irradiated mice, without antibiotics, showed exponential growth (Fig. 1). The effects of erythromycin, benzylpenicillin, and rifampin on *S. aureus* incubated in serum from normal and irradiated mice were similar (Fig. 1). At the concentrations used, the decrease in the number of CFU was close to the maximal decrease obtainable with the respective antibiotics.

Effect of irradiation on number of leukocytes. The numbers of granulocytes, lymphocytes, and monocytes determined before infection declined gradually during the first 5 days after irradiation to levels below 10% of the initial value (on day 0).

Relation between number of CFU at the site of infection and number of granulocytes in peripheral blood. (i) Without antibiotic treatment. The number of CFU per thigh 5 h after infection of saline-treated animals exhibited a close correlation ($r = 0.807$; $P < 0.001$) with the number of granulocytes in peripheral blood immediately before infection (Fig. 2); a 50% decrease in the number of granulocytes was accompanied by a 2.4-fold increase in the number of CFU. The numbers of lymphocytes and monocytes were also closely correlated with the number of CFU ($r = 0.683$, $P < 0.001$ and $r = 0.669$, $P < 0.001$, respectively), but multiple regression analysis showed that the contribution of the covariation of the number of CFU with the number of lymphocytes or

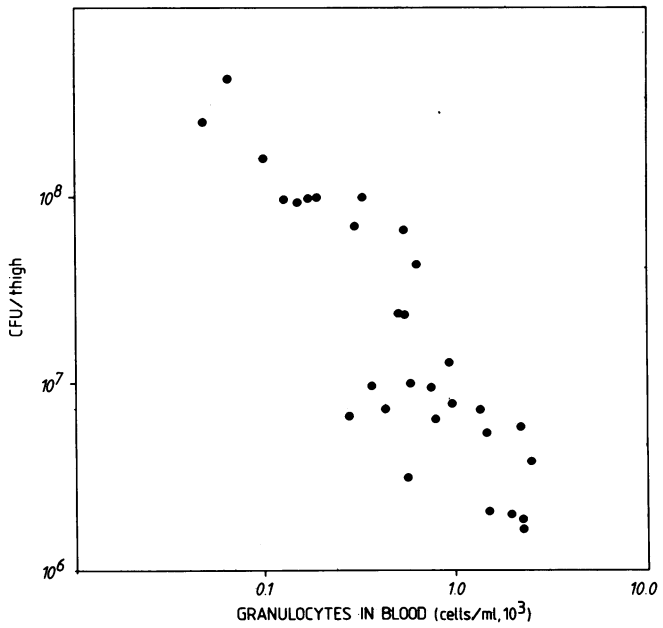

FIG. 2. Number of *S. aureus* CFU in an experimental thigh infection in relation to the number of granulocytes in peripheral blood.

monocytes was not significant ($0.1 < P < 0.2$) when the covariation with the number of granulocytes was taken into account.

(ii) With antibiotic treatment. At the dosage used, the following peak plasma concentrations were obtained: at a dose of 20 mg of erythromycin per kg, 2.24 (± 0.18) μg/ml and at a dose of 40 mg/kg, 4.37 (± 0.30) μg/ml; at a dose of 8 mg of benzylpenicillin per kg, 4.30 (± 0.50) μg/ml and at a dose of 16 mg/kg, 5.89 (± 0.81) μg/ml; at a dose of 0.25 mg of rifampin per kg, 0.118 (± 0.006) μg/ml and at a dose of 0.5 mg/kg, 0.229 (± 0.051) μg/ml. At the lowest dosages of all three antibiotics, the concentrations were too low to be determined. Tissue concentrations at the end of the experiments were below the level of detection.

For the experiments involving antibiotic treatment, only the relation between the number of granulocytes and the number of CFU per thigh was analyzed because the results of experiments without antibiotics had shown that lymphocytes and monocytes do not contribute statistically to the effect of granulocytes on the number of CFU. At each dosage (five animals) a close correlation was found between the number of CFU per thigh and the number of granulocytes, but the dose-effect relation for the individual antibiotics was different. A quantitative relation between the factors involved (granulocytes and antibiotics) was established by multiple-regression analysis using log antibiotic dose in milligrams per kilogram (DOS), log number of granulocytes per cubic millimeter (GRAN), and their statistical interaction (DOS × GRAN) as independent variables and the log number of CFU per thigh (CFU) as the dependent variable.

The pertinent regression equation is:

$$\text{CFU} = a_1 \times (\text{DOS}) + a_2 \times (\text{GRAN}) + a_3 \times (\text{DOS} \times \text{GRAN}) + a_4 \quad (1)$$

in which a_1, a_2, and a_3 are regression coefficients and a_4 is the intercept with the y axis, i.e., the virtual log dose value of 0. The values found for a_1 to a_4 are given in Table 1 for the three antibiotics. Equation 1 can also be written as:

$$\text{CFU} = (a_1 + a_3 \times \text{GRAN}) \times \text{DOS} + (a_2 \times \text{GRAN} + a_4) \quad (2)$$

Therefore, a_3 represents the change in the slope of the dose-effect relation caused by changes in the number of granulocytes. For rifampin and benzylpenicillin the calculated value of a_3 differed significantly from 0; for erythromycin, a_3 was much smaller and not significant, but the effect of granulocytes on the dose-effect relation (a_2) was still significant (Table 1). The other regression coefficients are not particularly informative because the position of the line of regression for the number of CFU versus dose is always dependent on the number of granulocytes: the slope is determined by a_1, a_3, and the log number of granulocytes (which is never equal to 0), while the intercept is given by a_2, a_4, and the log number of granulocytes. Moreover, the intercept represents the log number of CFU at a dose of 1 mg/kg, which is beyond the dosage range studied in all three cases. No consistent difference between real data and values calculated with the regression equation could be detected. In any case there was no indication that a critical number of granulocytes exists: the decrease in efficacy of the antibiotics between normal and very low numbers of granulocytes was continuous, as it was in animals not treated with antibiotics.

TABLE 1. Parameters of the effects of granulocytes and antibiotics on the number of *S. aureus* CFU in an experimental infection[a]

Antibiotic	Regression coefficients			Intercept (a_4)	Residual error (log CFU)
	a_1	a_2	a_3		
Rifampin	−6.82 (1.15)	0.66 (0.28)	1.65 (0.41)	3.49	0.35
Benzylpenicillin	−2.16 (0.66)	−0.84 (0.23)	0.56 (0.24)	9.54	0.24
Erythromycin	−0.60 (0.34)	−0.71 (0.16)	0.107 (0.12)	9.23	0.16

[a] According to the equation: log CFU = (a_1 + a_3 × log granulocytes) × log dose + (a_2 × log granulocytes + a_4). Parentheses indicate standard error.

These results are not suitable for presentation as a two-dimensional graph, because the number of granulocytes influences the effect of the antibiotic dose. Therefore, a similar multiple-regression analysis was performed using the number of days after irradiation instead of the log number of granulocytes. The mean value for the five animals in each dosage group is shown in Fig. 3. The increase in steepness of the dose-effect slope was significant for benzylpenicillin and rifampin and small and not significant for erythromycin. The upward shift in position was significant for all three antibiotics. In this figure the data for day 0 represent nonirradiated animals. Comparison with control values, e.g., the number of CFU without antibiotic treatment, determined for each consecutive day shows that in general the antibiotics were more effective than saline. Although the actual numbers of CFU differed from those in the experiment described in the previous paragraph, it can be seen that the number of CFU in controls increased on consecutive days after irradiation.

DISCUSSION

The present results show that in granulocytopenic mice a higher dosage of the bacteriostatic antibiotic erythromycin must be used to obtain the same effect as in normal mice; for the bactericidal antibiotics benzylpenicillin and rifampin, however, there is a dosage at which the number of granulocytes in the peripheral blood does not influence the ultimate effect, i.e., a reduction in the number of *S. aureus* at the site of infection. It was also found that monocytes and lymphocytes do not contribute to the antibacterial effect of antibiotic agents.

In this infection model the cellular exudate at the site of infection contained mainly granulocytes; granulocytopenic animals have fewer granulocytes at the site of infection than normal animals (8). However, it is virtually impossible to assess quantitatively the contribution of the number of granulocytes at the site of infection to inhibition of the proliferation of *S. aureus*. From our results it appears that the number of blood granulocytes is a parameter of the effect of granulocytes at the site of infection, probably because the granulocytes in an inflammatory exudate originate from the circulation. Disturbance of the production of granulocytes by irradiation leads within 5 days to a gradual decrease in the number of granulocytes to less than 10% of the initial number of cells. Since irradiation does not cause changes in serum factors, which could have an important effect on bacterial growth or antibiotic activity (2, 4), differences in bacterial growth or antibacterial efficacy must be attributed to changes in the number of leukocytes. Statistical analysis indeed showed a strong correlation between the number of granulocytes in peripheral blood and the number of bacteria

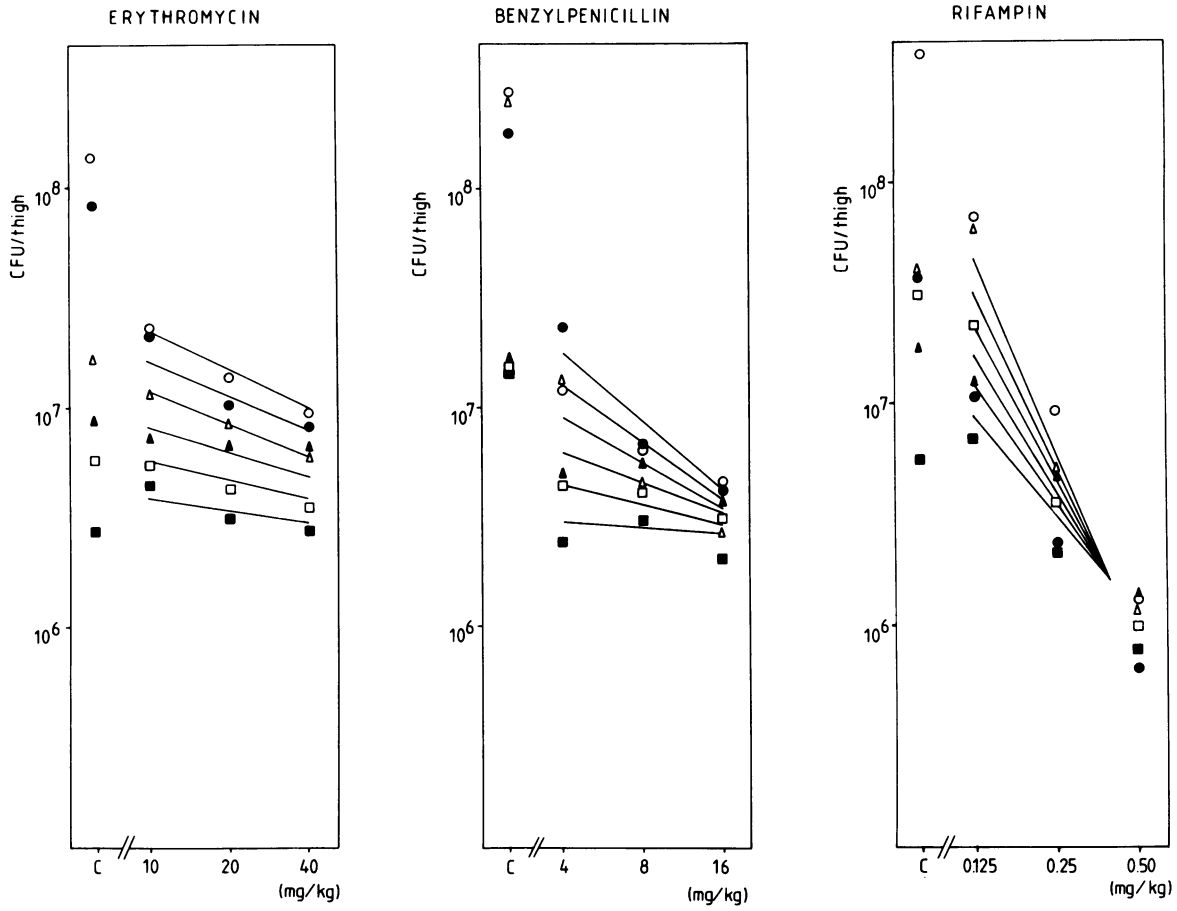

FIG. 3. Number of *S. aureus* CFU in an experimental thigh infection induced on 5 consecutive days after irradiation and treated with antibiotics; controls were not treated with antibiotics (C). Symbols: ■, day 0; □, day 1; ▲, day 2; △, day 3; ●, day 4; ○, day 5. Each point is the mean for five mice.

at the site of infection and showed no significant contribution from lymphocytes and monocytes in the circulation.

Previous studies from our laboratory on gram-negative bacterial infections in the same animal model showed that other beta-lactam antibiotics present a picture similar to that of benzylpenicillin and rifampin in the present study, i.e., a decreasing difference between the number of CFU in normal and irradiated animals at increasing dosages (11, 14), while aminoglycosides are characterized by a more or less parallel shift in the dose-effect relation for normal and irradiated animals, like erythromycin in the present study. On the other hand, the slope of the dose-effect relationship for aminoglycosides was much steeper than that for erythromycin. It should be noted that in our short-term infection model the maximal effect of antibiotics on the number of *S. aureus* is rather modest, as it is in vitro (Fig. 1). In fact, the highest dosages of all three antibiotics studied yielded practically the maximum effect under the conditions of the model infection, while even the lower dosages produced peak plasma concentrations that far exceeded the concentrations that are maximally effective in vitro. Free concentrations in muscle tissue follow the course of the plasma concentrations closely (H. Mattie, J. J. Hoogeterp, and J. Hermans, J. Pharmacokinet. Biopharm., in press). For all of these reasons there is no simple explanation for the striking difference in the interaction between granulocytes and antibiotics found for erythromycin on the one hand and penicillin and rifampin on the other.

The effect of antibiotic treatment on the number of *S. aureus* at the site of infection decreased when the number of granulocytes decreased, but not to the same extent for the three antibiotics. The results of multiple regression analysis provided some information about the quantitative aspects of this interaction and showed that when the number of granulocytes drops, a higher dosage of the antibiotic must be given to obtain the same effect. For the purpose of illustration, the calculated regression equations yield the following values: if the granulocyte count decreases from 200 to 100/mm^3, the dose of rifampin should be increased by 9%, that of benzylpenicillin should be increased by 31%, and that of erythromycin should be increased by 140%. Since there is also a significant interaction between penicillin or rifampin and granulocytes, an estimate can be made of the dose at which granulocytes no longer contribute to the antibacterial effect. This dose is 0.40 mg/kg for rifampin and 21.5 mg/kg for benzylpenicillin. This dose cannot be calculated for erythromycin because of the nearly parallel course of the dose-effect curves at different granulocyte levels.

The aim of this study was not to elucidate the relation between activity in vitro and that in vivo. Previous studies have shown that neither MIC (or MBC) nor killing rate in vitro is a good predictor of bactericidal or bacteriostatic

concentrations in vivo, not even in a simple experimental model (9). However, to relate our findings in vivo to the clinical situation in humans it is important that the plasma concentrations induced by the dosages used in our experiments lie in the range of the concentrations obtained with clinically therapeutic dosages (1). Although erythromycin may not be the drug of choice for staphylococcal infections in granulocytopenic patients, it is administered to patients who are allergic to penicillins. The present results indicate that a decrease in the number of granulocytes necessitates a considerable increase in the dosage of erythromycin to achieve the same effect as in patients with a normal number of granulocytes. The doses of benzylpenicillin and rifampin used in clinical practice are usually so high that the concentrations reached may abolish the negative effect of granulocytopenia. Since this study was not designed to establish all of the factors required to cure an infection, our results do not imply that granulocytes are not needed for final eradication of *S. aureus* in patients treated with benzylpenicillin or rifampin. Our findings at least give an indication of the consequences of granulocytopenia in relation to various dosages of the antibiotic agents.

LITERATURE CITED

1. **Acocella, G.** 1983. Pharmacokinetics and metabolism of rifampin in humans. Rev. Infect. Dis. **5**(Suppl. 3):428–432.
2. **Alexander, W. J., C. G. Cobbs, and M. R. Curtis.** 1980. Modification of bacterial serum susceptibility by rifampin. Infect. Immun. **28**:923–926.
3. **Brook, I., T. J. MacVittie, and R. I. Walker.** 1984. Recovery of aerobic and anaerobic bacteria from irradiated mice. Infect. Immun. **46**:270–271.
4. **Fitzgerald, S. P., and M. J. Rogers.** 1980. Bacteriostatic effect of serum: role of antibody to lipopolysaccharide. Infect. Immun. **27**:302–308.
5. **Gordon, L. E., D. Ruml, H. J. Dahne, and C. P. Miller.** 1955. Studies on the susceptibilities to ionizing irradiation. IV. The pathogenesis of the endogenous bacteremia in mice. J. Exp. Med. **102**:413–424.
6. **Hahn, H.** 1983. Antibacterial defence mechanisms. Infection **11**:112–118.
7. **Herbert, W. J.** 1978. Laboratory animal techniques for immunology, p. A4.14–A4.15. *In* D. M. Weir (ed.), Handbook of experimental immunology 1978, 3rd ed. Blackwell Scientific Publications, Ltd., Oxford.
8. **Kunst, M. W., H. Mattie, and R. van Furth.** 1979. Antibacterial efficacy of cefazolin and cephradine in neutropenic mice. Infection **7**:30–34.
9. **Mattie, H.** 1981. Kinetics of antimicrobial action. Rev. Infect. Dis. **3**:19–27.
10. **Mattie, H., W. R. O. Goslings, and E. L. Noach.** 1973. Cloxacillin and nafcillin: serum binding and its relationship to antibacterial effect in mice. J. Infect. Dis. **128**:170–178.
11. **Mattie, H., and G. B. van der Voet.** 1981. The relative potency of amoxycillin and ampicillin in vitro and in vivo. Scand. J. Infect. Dis. **13**:291–296.
12. **Meddens, M. J. M., J. Thompson, H. Mattie, and R. van Furth.** 1985. Role of granulocytes and monocytes in the prevention and therapy of experimental *Staphylococcus epidermidis* endocarditis in rabbits. J. Infect. **11**:41–50.
13. **Miller, C. P.** 1951. The role of infection in irradiation injury. J. Lab. Clin. Med. **38**:331–343.
14. **van der Voet, G. B., H. Mattie, and R. van Furth.** 1983. The antibacterial activity of combinations of mecillinam and ampicillin in vitro and in normal and granulopenic mice. Scand. J. Infect. Dis. **15**:91–96.
15. **van der Voet, G. B., H. Mattie, and R. van Furth.** 1984. Quantitative determination of the effect of granulocytes on the course of experimental infections during antibiotic treatment. Infection **12**:5–9.
16. **van der Voet, G. B., H. Mattie, and R. van Furth.** 1985. Comparison of the antibacterial activity of azlocillin and ticarcillin in vitro and in irradiated neutropenic mice. J. Antimicrob. Chemother. **16**:605–613.
17. **van Furth, R., and Z. A. Cohn.** 1968. The origin and kinetics of mononuclear phagocytes. J. Exp. Med. **128**:415–435.