The Effect of Antibiotics on *Escherichia coli* Ingested by Macrophages[1] (37173)

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It has been shown that bacteria may survive ingestion by macrophages despite the bactericidal properties of these phagocytic cells (1). Rous and Jones (2) demonstrated that intracellular bacteria are protected from the lethal actions of bactericidal drugs such as cyanide. More recently several investigators have demonstrated that the intracellular environment also protects bacteria from antibiotics (3, 4). Since previous studies showed that rifampin could kill staphylococci that had been ingested by polymorphonuclear neutrophils (5) we performed experiments to study the ability of rifampin to eradicate *E. coli* from macrophages.

Materials and Methods. Bacteria. An overnight culture of *Escherichia coli* (strain 0111B4) in trypticase soy broth was washed and diluted in 0.9% saline to a final concentration of 10^5 bacteria/ml. *E. coli* 0111B4 antiserum (Difco Laboratories, Detroit, Michigan) was dialyzed overnight against distilled water to remove preservatives; 10^5 *E. coli* were incubated with 5% antiserum in 1.0 ml of 0.9% saline for 2 hr in order to opsonize the bacteria.

Antibiotics and antibiotic sensitivity. Diagnostic standard powders of rifampin, ampicillin and gentamicin were utilized. Assays of antibiotic sensitivity of the bacteria were done by standard serial dilution techniques with 10^5 organisms per tube. The minimal bactericidal concentration (MBC) was defined as the lowest concentration of antibiotics with which there was no growth in a 0.1-ml sample plated on trypticase soy agar.

Glassware. Coverslips were washed in Microsolve detergent (Microbiological Associates, Inc., Bethesda, Maryland), rinsed 12 times in tap water, 4 times in distilled water and then autoclaved. These were then carefully introduced into Leighton tubes using aseptic techniques. All other glassware was siliconized.

Macrophages. Peritoneal macrophages were obtained from white, 15–20-g male mice (Strain DUB/ICR, Dublin, Virginia, Institute for Cancer Research). Mice were killed by cervical dislocation and the peritoneal cavity was washed with 5 ml of Medium 199 (Microbiological Associates, Inc., Bethesda, Maryland) at 4°. Pooled peritoneal aspirates from 5 mice were centrifuged at 500*g* for 8 min at 4° and the resulting cell button was resuspended in 5 ml of Medium 199 containing 20% fetal bovine serum. One milliliter of this suspension (containing 1–3 $\times$ 10^6 mononuclear phagocytes) was layered on coverslips within Leighton tubes and incubated for 72 hr in 5% CO_2 in air at 37°. The cell culture medium was changed after 24 and 72 hr of incubation.

Human macrophages were obtained from peripheral venous blood by methods previously described (6). Heparinized blood was sedimented with 6% dextran for one hour. The leukocyte button, obtained by centrifuging the supernatant was resuspended in 7.2 ml of Medium 199 containing 120 units of heparin and 4.8 ml of fetal bovine serum. Two milliliters of this suspension were put

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into Leighton tubes containing coverslips and incubated in 5% CO_2 in air at 37° for 72 hr. The media were changed after 24 and 72 hr of incubation.

Effect of antibiotics on intracellular E. coli. Immediately after the 72-hr media change, 10^4 viable, opsonized *E. coli* were introduced into Leighton tubes with either human or mouse macrophages. After 2 hr of incubation, phagocytosis of bacteria could be demonstrated in Giemsa stained coverslip preparations; 100 μg of either ampicillin, gentamicin or rifampin were then added to the tubes. The macrophage preparations with antibiotics were incubated for 18 more hr and control tubes with no antibiotics were incubated simultaneously. At the end of this time, the supernatant was decanted and viable bacteria were quantitated by serial dilution and pour-plate techniques. The cover slips were carefully removed, washed in 0.9% saline and placed in new sterile Leighton tubes. Melted trypticase soy agar at 42° was pipetted into the tubes and after overnight incubation at 37°, bacterial colonies were enumerated using a dissecting microscope.

Results. The results are summarized in Table I. All bacteria were killed in experiments performed with frozen–thawed macrophages plus antibiotics, indicating that intact macrophages are necessary for protection of bacteria from the lethal actions of the antibiotics. In control tubes containing macrophages but no antibiotics, viable bacteria were present both in the media and on the coverslips.

The minimal bactericidal concentrations of ampicillin, gentamicin and rifampin for *E. coli* (0111B4) were 1.56 μg/ml, 0.78 μg/ml, and 6.25 μg/ml, respectively.

Discussion. Several investigators have been concerned with the problem of the effect of antimicrobial agents on bacteria inside macrophages. Prior to the present studies no antimicrobial agent has been shown to kill bacteria in macrophages after relatively short (24 hr) incubation. Mackaness (1) demonstrated that rabbit peritoneal macrophages protected *Mycobacterium tuberculosis* from the bactericidal actions of streptomycin. Hart *et al.* (7) showed with electron microscopy that most mycobacteria ingested by macropages appeared intact after incubation with streptomycin. Shaffer *et al.* (4) studied brucella that had been ingested by rat peritoneal phagocytes and found that concentrations of streptomycin as great as 50,000 μg/ml were unable to kill intracellular organisms.

Streptomycin suppressed intracellular growth of bacteria after prolonged (more than 4 weeks) incubation with macrophages that had ingested *Mycobacterium lepraemurium* (8). Bonventre *et al.* (9, 10) showed that mouse peritoneal macrophages accumulate radio-labeled dihydrostreptomycin only after

TABLE I. Survival of *E. coli* within Macrophages Incubated with Antibiotics.[a]

Antibiotics	Media (Extracellular bacteria)	Coverslip (Cell-associated bacteria)	No. of experiments
	Mouse peritoneal macrophages		
100 μg ampicillin	0	292 ± 12	21
100 μg gentamicin	0	254 ± 34	21
100 μg rifampin	0	0	21
No antibiotics	$>10^6$	602 ± 22	12
	Human macrophages		
100 μg ampicillin	0	143 ± 24	9
100 μg gentamicin	0	87 ± 12	9
100 μg rifampin	0	0	9
No antibiotics	$>10^6$	619 ± 24	6

[a] 10^4 opsonized *E. coli* were incubated with 10^6 macrophages for 2 hr; 100 μg of antibiotics were then added and incubation was continued for 18 more hours. Bacterial counts were determined in the media (extracellular bacteria) and on the coverslip (macrophage-associated bacteria). Means ± standard error of the mean are shown.

prolonged culture.

Previous studies in our laboratory (5) demonstrated the ability of rifampin to kill staphylococci that survived phagocytosis by polymorphonuclear neutrophils. Survival of bacteria inside macrophages is a well-known phenomenon in diseases such as tuberculosis, brucellosis and salmonellosis. Since rifampin is a very effective antituberculosis agent (and perhaps the only one that can completely sterilize organs of tuberculous mice after relatively short therapy (11)) we feel that the ability of the antibiotic to kill bacteria inside macrophages *in vitro* correlates with its efficacy in the treatment of experimental tuberculosis. The bactericidal activity of an antibiotic towards intracellular microbes may be related to its penetration of phagocyte membranes. Since rifampin is a highly lipid soluble substance this may explain its unique ability to kill intracellular organisms.

Summary. Macrophages obtained from mouse peritoneal washings or the culture of human peripheral monocytes, were incubated *in vitro* with opsonized *E. coli*. Macrophages containing viable *E. coli* were then incubated with either rifampin, gentamicin or ampicillin. Only rifampin killed all intracellular and extracellular bacteria whereas gentamicin and ampicillin eradicated only extracellular bacteria. Rifampin is able to penetrate macrophages and kill bacteria that have survived phagocytosis.

1. Mackaness, G. B., J. Pathol. Bacteriol. **LXIV**, 429 (1952).

2. Rous, P., and Jones, F. S., J. Exp. Med. **23**, 601 (1916).

3. Magoffin, R. L., and Spink, W. W., J. Lab. Clin. Med. **37**, 924 (1951).

4. Shaffer, J. M., Kucera, C. J., and Spink, W. W., J. Exp. Med. **97**, 77 (1953).

5. Mandell, G. L., and Vest, T. K., J. Infect. Dis. **125**, 486 (1972).

6. Mandell, G. L., and Hook, E. W., Amer. J. Med. **47**, 473 (1969).

7. Hart, P. D., Armstrong, J. A., Brown, C. A., and Draper, P., Infec. Immunity **5**, 803 (1972).

8. Chang, Y. T., Appl. Micro. **17**, 750 (1969).

9. Bonventre, P. F., Hayes, R. and Imhoff, J., J. Bacteriol. **93**, 445 (1967).

10. Bonventre, P. F., and Imhoff, J. G., Infec. Immunity **2**, 89 (1970).

11. Batten, J., Tubercle **50**, 294 (1969).

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