

Activities of Antimicrobial Agents against Intracellular Pneumococci

GERALD L. MANDELL* AND ELIZABETH J. COLEMAN

Division of Infectious Diseases, University of Virginia Health Systems, Charlottesville, Virginia 22908

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Pneumococci can enter and survive inside human lung alveolar carcinoma cells. We examined the activity of azithromycin, gentamicin, levofloxacin, moxifloxacin, penicillin G, rifampin, telithromycin, and trovafloxacin against pneumococci inside and outside cells. We found that moxifloxacin, trovafloxacin, and telithromycin were the most active, but only telithromycin killed all intracellular organisms.

In 1916 Rouss and Jones showed that living, but not dead, phagocytes could protect microbes from killing by antiserum and potassium cyanide (11). Though the pneumococcus is usually considered to be an extracellular pathogen, studies have indicated that pneumococci can enter and survive inside human lung alveolar carcinoma cells (type II pneumocytes, A549 cells) (14). The clinical relevance of this observation is not clear. Furthermore, pneumococci may persist in tissue sites despite in vitro sensitivities to antibiotics and adequate antimicrobial levels. A recent clinical study documented the persistence of *Streptococcus pneumoniae* in the middle-ear fluid of patients treated with several different antibiotics (2). The role of intracellular persistence in this phenomenon is unknown.

We studied several antimicrobial agents with good activity against the pneumococcus to determine their ability to kill the organism inside A549 cells. The agents studied included penicillin G and gentamicin (7, 10, 12), which remain largely extracellular, and azithromycin, levofloxacin, rifampin, trovafloxacin, telithromycin (3, 5, 8, 15), and moxifloxacin (9), all of which have been reported to penetrate cells well. The minimal bacteriostatic and bactericidal concentrations of each agent for *S. pneumoniae* 14.8 were determined in RPMI 1640 (the A549 culture medium; Biowhittaker, Walkersville, Md.).

Purified polymorphonuclear leukocytes were obtained from heparinized (10 U/ml; Lymphomed Fujisawa USA Inc., Deerfield, Ill.) human venous blood by a Ficoll-Hypaque separation procedure adapted from the work of Ferrante and Thong (4). Cells were resuspended in Hanks balanced salt solution (HBSS), counted using a hemocytometer, and found to be 95% pure polymorphonuclear leukocytes. *Micrococcus luteus* ATCC 9341 (American Type Culture Collection, Rockville, Md.) and *Staphylococcus aureus* ATCC 27217 were cultured in tryptic soy broth (Difco, Detroit, Mich.) or on 5% sheep blood agar (Becton Dickinson, Cockeysville, Md.). *S. pneumoniae* 14.8 was supplied by Daniel Musher, Infectious Disease Section, Veterans Administration Medical Center, Houston, Tex. A549, a human lung alveolar carcinoma (type II pneumocyte) cell line, was obtained from ATCC. Cells were grown in RPMI 1640 (Biowhittaker) with 10% fetal calf serum (Biowhittaker), 25 mM HEPES (Sigma Chemical Company, St. Louis, Mo.), and 100 U of penicillin G per ml with 100 µg of streptomycin (Biowhittaker) per ml. A549 monolayers were grown to con-

fluence in 24-well tissue culture plates (Nalge Nunc International, Roskilde, Denmark) at 37°C with 5% CO₂.

Penicillin G and gentamicin sulfate were purchased from Sigma Chemical Company. Levofloxacin was provided by the R. W. Johnson Pharmaceutical Research Institute, Spring House, Pa. Stock solutions of penicillin G, gentamicin sulfate, and levofloxacin were made up in HBSS. Rifampin was purchased from Sigma Chemical Company, and a stock solution was made fresh daily by dissolving rifampin in methanol and diluting this further with HBSS. Trovafloxacin was obtained from Pfizer Pharmaceuticals, New York, N.Y. Telithromycin was supplied by Hoechst-Marion-Roussel, Romainville, France. Stock solutions of trovafloxacin and telithromycin were made by resuspending powder in 1% HCl and sterile water. Moxifloxacin was obtained from Bayer Corporation, West Haven, Conn., and stock solutions were made by resuspending the drug in sterile water. Azithromycin was provided by Pfizer Pharmaceuticals and a stock solution was made by initially dissolving azithromycin in ethanol and then diluting this with HBSS.

In order to make meaningful comparisons of antimicrobial agent activity, we utilized in vitro antibiotic concentrations (except with gentamicin and rifampin) similar to peak concentrations in serum, as reported in the literature (1, 13, 16). In some cases lower concentrations were also used.

The MICs and minimum bactericidal concentrations (MBCs) for *S. pneumoniae* were determined by a broth dilution method using a concentration of 5×10^5 CFU/ml in RPMI 1640 (6). Incubation was carried out at 37°C for 24 h, and growth of any viable organisms was checked by using a 10-µl loop to inoculate the organisms onto blood agar.

Cell cultures infected with intracellular pneumococci were prepared as follows. A549 monolayers grown to confluence were washed to remove penicillin G and streptomycin from the growth media. *S. pneumoniae* 14.8 was cultured for 16 to 18 h in Todd-Hewitt broth (Difco) supplemented with 0.5% yeast extract (Difco) and 0.25% choline bitartrate salt (Sigma) and was prepared by centrifugation and washing of the pellet in normal saline. The pellet was then resuspended in HBSS, and the optical density at a wavelength of 580 nm was adjusted to 1.10, which was indicative of 1×10^9 CFU/ml. Samples of 9×10^7 CFU of *S. pneumoniae* 14.8 were incubated for 2 h at 37°C with A549 monolayers in 1 ml of A549 growth medium (omitting the penicillin G and streptomycin) per well. Monolayers containing intracellular pneumococci were then washed with HBSS. A549 growth medium containing gentamicin (50 µg/ml) was added to each well and incubated for 2 h at 37°C to kill extracellular pneumococci. Cultures showed $12,572 \pm 2,609$

* Corresponding author. Mailing address: University of Virginia Health Systems, P.O. Box 801341, Division of Infectious Diseases, Charlottesville, VA 22908-1341. Phone: (804) 924-5942. Fax: (804) 982-0002. E-mail: gm@virginia.edu.

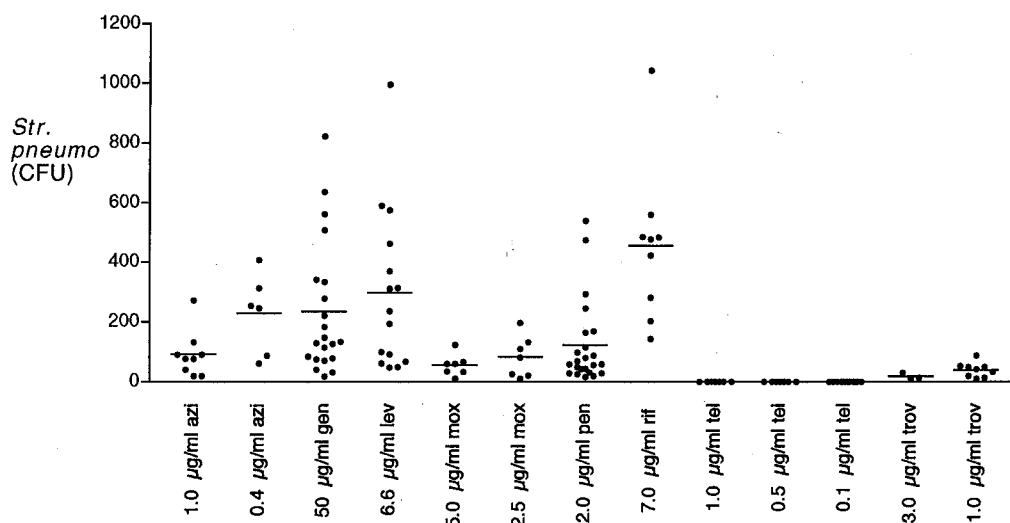


FIG. 1. Growth of pneumococci in the presence of antibiotics. Details of the experiment are given in the text. Azi, azithromycin; gen, gentamicin; lev, levofloxacin; mox, moxifloxacin; pen, penicillin G; rif, rifampin; tel, telithromycin; trov, trovafloxacin.

(standard error of the mean, $n = 8$) CFU after the gentamicin incubation. The monolayers were then washed with HBSS. A549 growth medium with or without antibiotic was added and incubated with the A549 cells containing pneumococci for 18 h. Trypan blue staining of A549 cells showed that in the absence of antibiotics all cells were nonviable and that with all antibiotics tested more than 98% of the A549 cells were viable. After incubation, the supernatants were serially diluted and plated onto chocolate agar, and the monolayers were washed with HBSS to remove the antibiotics. One hundred microliters of trypsin-EDTA (Sigma Chemical) was added per well and incubated for 10 min at 37°C to lift the monolayers, which were then lysed by the addition of 900 μ l of sterile water to each well for 10 min at 37°C. Viable organisms were enumerated by serial dilutions and counts of CFU on chocolate agar.

Those monolayers incubated without antibiotics showed "too numerous to count" growth of pneumococci, indicating that the penicillin G and streptomycin present in the original A549 growth medium had no significant effect on bacterial growth.

Table 1 shows the MICs and MBCs of the antibiotics used against *S. pneumoniae* 14.8. Results of the assays to determine the ability of antibiotics to kill intracellular pneumococci are shown in Fig. 1. In all experiments, supernatant cultures showed no growth. Despite concentrations of gentamicin, penicillin G, and rifampin far exceeding the MBCs in A549 growth medium, significant numbers of pneumococci survived inside

the cells after 18 h of incubation. Data were analyzed using a Mann-Whitney rank sum nonparametric test. All of the antimicrobial agents tested were more active than penicillin G in killing intracellular organisms ($P < 0.05$). Telithromycin was most active ($P < 0.01$) and was unique in that it consistently sterilized the cultures, even at low extracellular concentrations. In more than 100 experiments, the only agent that sterilized the cells was telithromycin. Experiments were performed with 2- and 8-h incubation times (data not shown), and pneumococcal survival decreased in a linear fashion with no difference in the rank order of effectiveness.

In order for an antimicrobial agent to kill intracellular organisms, it must enter the cell and the compartment where the microbe resides at concentrations above the MBC for that milieu. Ideally, an antibiotic should eradicate pathogens from tissue sites, including intracellular locations. This may be more important for some organisms than others. We know, for example, that beta-lactam antibiotics failed to cure patients with Legionnaires' disease, despite the causative organism's susceptibility to these agents in vitro. The presumed explanation is that the intracellular location of the organism protected it from the action of the antibiotic. Agents that do get into cells, such as macrolides and fluoroquinolones, are effective against this disease.

Diseases caused by extracellular pathogens such as the pneumococcus clearly respond to therapy with agents, such as penicillin G, which penetrate cells poorly. Only a small proportion (about 8%) of the pneumococci incubated with A549 cells were found to be viable after incubation for 18 h with the extracellular antibiotic penicillin G. However, it may be advantageous to destroy organisms in intracellular sites to reduce relapse, recolonization, and possibly the development of resistance. Clinical studies to prove this hypothesis will be difficult to perform because of the variables associated with antimicrobial agents of different classes.

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TABLE 1. MICs and MBCs of various antibiotics against *S. pneumoniae* 14.8 in RPMI

Antibiotics	MIC (μ g/ml)	MBC (μ g/ml)
Azithromycin	0.0006	0.02
Gentamicin	1.56	6.25
Levofloxacin	0.6	2.5
Moxifloxacin	0.08	0.31
Penicillin G	0.01	0.01
Rifampin	0.05	1.56
Telithromycin	0.0002	0.001
Trovafloxacin	0.125	0.5

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