Antibiotic Control of *Mycoplasma* in Tissue Culture

D. PERLMAN, SHARON B. RAHMAN, AND JOAN B. SEMAR

Squibb Institute for Medical Research, New Brunswick, New Jersey

Received for publication 8 August 1966

**ABSTRACT**

Seven of eight strains of *Mycoplasma* (PPLO) were found to be sensitive to the deoxystreptamines, certain macrolides, and the tetracyclines. These antibiotics are relative noncytotoxic. Kanamycin and tetracycline were useful in eliminating PPLO (pleuropneumonia-like organisms) strain Squibb no. 1 from a HeLa cell line which was deliberately contaminated with PPLO. Repeated exposure of *M. laidlawii* type B cells to neomycin resulted in a 50-fold increase in resistance, and the resistant strain was also resistant to gentamicin, kanamycin, neomycin, and paromomycin. A tetracycline-resistant strain of this culture was found to be resistant to 7-chlor-tetracycline, 7-chlor-6-demethyltetracycline, and 5-hydroxytetracycline. One PPLO strain, Squibb no. 2, derived from a contaminated HeLa cell culture, was resistant to all antibiotics studied.

Biochemists and microbiologists using cell cultures as biochemical systems have become increasingly aware of the problems introduced by contaminating microorganisms. Although antibiotics have been widely used to eliminate bacterial, fungal, and yeast contamination, comparatively little attention has been paid to *Mycoplasma* (PPLO) contamination. Among the methods suggested for eliminating these parasites from cell lines are heat treatment (8), use of specific antiserum (14), and antibiotic treatment. Among the antibiotics used in various laboratories were tetracycline (3, 9), kanamycin (5, 15), tylosin (6), 7-chlortetracycline (7), and a mixture of chloramphenicol and novobiocin (2).

Although Carski and Sheppard (3) reported elimination of *Mycoplasma* from cell lines cultivated through four successive passages in the presence of only 2.5 μg/ml of tetracycline, Hooser et al. (10) found it necessary to use 20 μg/ml. Gori and Lee (7) noted that tetracycline treatment even at cytotoxic doses for periods up to 5 months proved capable only of depressing the severity of the infection without effecting sterilization. In a similar situation, Friend et al. (6) found that several chemotherapeutic agents, including tetracycline, kanamycin, erythromycin, chloramphenicol, polymyxin, stovarsoi, and iodine, frequently "cured" the infected cultures when examined immediately after treatment, only to have the pleuropneumonia-like organisms (PPLO) reappear in the subsequent passage.

It seems likely that, with continued use of "PPLO suppressive" antibiotics in tissue culture media, there will eventually emerge "antibiotic-resistant" *Mycoplasma* strains. Strains resistant to kanamycin have already been described (2, 16), and a strain resistant to many antibiotics has been found as a tissue culture contaminant (2).

We have examined the antibiotic sensitivity of eight *Mycoplasma* strains in vitro and have used several of the most effective of these antibiotics to control *Mycoplasma* infections in cell lines. We have also studied the problem of "induced antibiotic resistance" in *Mycoplasma* strains with the objective of determining whether antibiotic cross-resistance is found in these organisms as it is found in bacteria.

**MATERIALS AND METHODS**

The eight *Mycoplasma* strains used in this study included: *M. laidlawii* type B; *M. arthritis* strain L; *M. gallisepticum* strain A-5969-TC (from two sources); *M. hominis*, strains Campo W and 39-S4; and two strains from infected HeLa tissue cultures, designated Squibb no. 1 and Squibb no. 2. Difco PPLO Enrichment Broth (without crystal violet) supplemented with yeast extract, sodium acetate, and PPLO Serum Fraction (Difco) was used for propagating the identified *Mycoplasma* species. The Squibb cultures 1 and 2 were maintained in soy-peptone broth supplemented with yeast extract, NaCl, and human serum as mentioned by Kenny and Pollock (11). Difco PPLO Agar (with PPLO Serum Fraction) was used for the antibiotic sensitivity tests for the named species, and Pollock's (11) medium (with Noble agar) was used for the Squibb cultures.

Antibiotic sensitivity tests were carried out by use of an agar dilution method. Dilutions of the antibiotic test solution in PPLO Agar were added to petri plates...
The sensitivity to amphomycin, test), of coplasma in test. Cultures. Castrejon-Diez exhibited by the PPLO in inhibitory to some these infection with Squibb et al. All of the antibiotics studied in this tissue culture agreement, too. Kanamycin (at 200 ppm) and tetracycline (at 10 ppm) were effective in eliminating the Squibb no. 1 strain of Mycoplasma from the HeLa cell line. The monolayers appeared to be free from PPLO after four treatments (medium change twice a week and harvest every 7 days). No recurrence of infection was noted when antibiotic treatment was discontinued for a 4-week period. On the other hand, neither of these two antibiotics nor any other was effective in freeing the HeLa cells from Squibb no. 2. We do not advise continued exposure of the tissue culture cells to any antibiotic for fear that an antibiotic-resistant Mycoplasma will develop (see below). (Note added in proof: further study has shown PPLO strain 2 to be sensitive to lincomycin at 20 ppm.)

Induced antibiotic resistance in Mycoplasma. The strain of M. laidlawii type B was initially found to be quite sensitive to kanamycin and neomycin (minimal inhibitory concentrations of the order of 10 μg/ml when the agar dilution test was used). When this culture was exposed to increasing concentrations of neomycin, resistant strains appeared. After eight exposures, a strain resistant to 500 μg/ml was obtained. This strain was found to be resistant to kanamycin, gentamicin, and paromomycin, though increase in resistance was not as marked (50-fold) as with neomycin. When tetracycline was used as the

Results and Discussion

Antibiotic sensitivity of Mycoplasma cultures. The eight Mycoplasma cultures were tested for sensitivity to 40 antibiotics by use of the agar dilution test. Only Squibb culture 2 was not inhibited by any of the antibiotics. The following 20 antibiotics were essentially inactive against Mycoplasma in vitro (produced no inhibition of growth of test strain at 100 μg/ml in agar dilution test): amphotericin B, bacitracin, benzylpenicillin, candidicidin A, cycloheximide, cyclodosate, eusorcin, filipin, griseofulvin, nystatin, oleandomycin, patulin, polymyxin, ristocetin, streptomycin, trichomycin, vancomycin, vernamycins A and B, and viomycin. Of these antibiotics listed as “ineffective,” a few were inhibitory to some of the seven susceptible PPLO cultures, but not to others. Studies with these antibiotics were discontinued, because we were only interested in antibiotics “active” against all the PPLO cultures. The following antibiotics were quite active in inhibiting the seven susceptible PPLO cultures: carbomycin, dactinomycin, streptomycin, tetracycline, and thiostrepton. Unfortunately, however, they are too cytotoxic (13) to be considered for use in eliminating PPLO from tissue culture.

The 15 antibiotics which were active against the seven susceptible PPLO test strains and had relatively “low” or “no” cytototoxicity are listed in Table 1. Several of these are stable (chemically) when dissolved in tissue culture media and incubated at 37 C, whereas others are rather rapidly inactivated. The useful antibiotics may be grouped on the basis of their chemistry as well as the response in our screen: (i) the deoxystreptamines: gentamicin, kanamycin, hygromycin B, neomycin, and paromomycin; (ii) the macrolides: erythromycin, spiramycin, and tylosin; (iv) the tetracyclines: 7-chlortetracycline, 6-demethyl-7-chlortetracycline, 5-hydroxytetracycline, and tetracycline. The members of the deoxystreptamine group and the macrolide group appear to have special advantages for use in eliminating PPLO contaminants from tissue cultures, because these antibiotics have high chemical stability in tissue culture media and low cytototoxicity. The tetracyclines are quite cytotoxic and, except for 6-demethyl-7-chlortetracycline, are not stable in tissue culture media at 37 C for any great length of time. In general, our results on antibiotic sensitivity of Mycoplasma agree with those reported by Arai et al. (1).
Table 1. Antibiotics useful in controlling PPLO contamination in tissue cultures

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Stability in tissue culture media</th>
<th>Conc showing marked cytotoxicity</th>
<th>Minimal conc inhibiting PPLO in agar streak test</th>
<th>Conc recommended for controlling PPLO in tissue cultures</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloramphenicol</td>
<td>High</td>
<td>μg/ml</td>
<td>μg/ml</td>
<td>μg/ml</td>
</tr>
<tr>
<td>7-Chloretetracycline</td>
<td>Very low</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6-Demethyl-7-chloretetracycline</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Erythromycin</td>
<td>High</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fusidic acid</td>
<td>High</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gentamicin</td>
<td>High</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-Hydroxytetracycline</td>
<td>Moderate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hygromycin B</td>
<td>Moderate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kanamycin</td>
<td>Very high</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neomycin B</td>
<td>Very high</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Novobiocin</td>
<td>Low</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Paromomycin</td>
<td>High</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spiramycin</td>
<td>Moderate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tetracycline</td>
<td>Moderate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tylosin</td>
<td>Moderate</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a Stability scale: half-life of 2 days, very low; half-life of 4 days, low to moderate; half-life of 8 days, very high.

b Data from Perlman and Brindle (12).

c As determined in twofold agar dilution test (see text).

d Recommended on basis of 3-day incubation period between medium changes.

antibiotic, a 40-fold increase in resistance was observed after seven passages. This tetracycline-resistant culture was considerably more resistant to 7-chloretetracycline and 5-hydroxytetracycline than was the parent culture. A 10-fold increase in resistance to chloramphenicol was also noted. The ease with which resistant cultures were obtained was ill for those who plan routinely to use certain antibiotics to eliminate Mycoplasma from their cell lines.

Acknowledgments

We are indebted to H. E. Morton, Irving Jaffe, and P. M. Arnow for Mycoplasma cultures used in this study.

Literature Cited

13. Perlman, D., N. A. Giuffre, P. W. Jackson,

