Effects of Rokitamycin and Other Macrolide Antibiotics on Mycoplasma pneumoniae in L Cells

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Fifty strains of Mycoplasma pneumoniae in L cells were tested for susceptibility to macrolide antibiotics. Rokitamycin, a new macrolide antibiotic, was most active against these organisms, with an MIC for 90% of strains of 0.007 μg/ml. The MICs of erythromycin, josamycin, and kitasamycin for 90% of strains were 0.03, 0.03, and ≥0.06 μg/ml, respectively. Based on these results, rokitamycin is a promising antibiotic for the treatment of mycoplasmal infections, and further clinical investigations are needed.

Mycoplasma pneumoniae is the causative agent of upper respiratory tract infections and pneumonia in humans (3, 5). For chemotherapy against M. pneumoniae infections, macrolide and tetracycline antibiotics have been widely used, because of the susceptibilities of M. pneumoniae to these antibiotics as assayed by the agar method (1, 10). Because the adherence of mycoplasmas to the epithelium of the respiratory tract is an initial step in successful colonization (2, 4, 6, 9), it might be relevant for the treatment of M. pneumoniae infections to examine the effects of antibiotics on the elimination of attached and colonized mycoplasmas from the surface of cells (1, 7, 13).

The purpose of this study was to determine the in vitro activities of macrolide antibiotics, including rokitamycin (RKM; TM-19Q), one of the acyl derivatives of 16-membered macrolides (11, 12), against recent clinical isolates of M. pneumoniae, comparing MICs determined by the agar dilution method and the minimum concentrations to eliminate M. pneumoniae from infected L cells.

Forty-nine strains of M. pneumoniae isolated from throat swabs of patients with pneumonia plus the standard M. pneumoniae strain, FH, were used. Isolates were subcultured fewer than five times in broth and stored at −80°C until use. The antibiotics used were erythromycin (EM; Shionogi Pharmaceutical Co. Ltd., Osaka, Japan), josamycin (JM; Yamanouchi Pharmaceutical Co. Ltd., Tokyo, Japan), and kitasamycin (leucomycin; LM) and RKM (Toyozo Co. Ltd., Tokyo, Japan).

Antimicrobial activity was determined by the agar dilution method. Briefly, each M. pneumoniae strain was grown in Hayflick PPLO liquid medium (Difco Laboratories, Detroit, Mich.). Serial twofold dilutions of the antibiotics to be tested were prepared in PPLO agar medium. The agar plates were inoculated with approximately 10⁵ CFU/10 μl by using a microinoculator. The plates were read after 6 days of incubation at 37°C. The MICs of the antibiotics were defined as the lowest concentrations that completely inhibited development of microscopically visible colonies on agar.

For adaptation of mycoplasmas to L cells, L cells (2 × 10⁶ per well) obtained by trypsinization from mycoplasma-free monolayer L cells were cultured in flat-bottom Microwell plates (Nunc Co. Ltd., Roskilde, Denmark) in Eagle minimum essential medium (Nissui Co. Ltd., Tokyo, Japan) fortified with 10% newborn calf serum and containing 2 mM L-glutamine and 100 U of penicillin per ml. After 24 h of incubation, the cells were infected with 10⁵ CFU of M. pneumoniae per ml in the liquid medium and incubated at 37°C in a CO₂ incubator for 7 days. The microorganisms in the supernatants of L cells were passaged more than five times, and the culture fluids were stored at −80°C until use.

To determine the abilities of the antibiotics to eliminate M. pneumoniae from L cells, L cells cultured for 24 h in Microwell plates were infected with 10⁶ CFU of M. pneumoniae per ml as described above. After 3 days of cultivation, twofold-diluted antibiotics were added to the cultures of M. pneumoniae-infected L cells. The plate was incubated at 37°C in a CO₂ incubator for 7 days. MICs of the antibiotics in M. pneumoniae-infected L cells were determined by plating 10 μl of cell suspensions obtained by freeze-thawing twice on an agar plate. In this cultivation, the number of mycoplasmas in cell-culture fluids without antibiotics reached 10⁶ CFU/ml.

For detection of M. pneumoniae in L cells, a DNA staining method was used as described previously (8). Briefly, 10⁵ L cells per cover slip (5 by 150 mm) with M. pneumoniae FH were fixed with acetic acid-methanol for 15 min, 5 days after infection, and then these cover slips were immersed with 0.1% of Hoechst stain 33258 (HoechstRoussel Pharmaceuticals Inc., Somerville, N.J.) and kept in a dark box for 30 min. The cover slips were rinsed three times with distilled water and mounted on microscope slides. Excitation of fluorescence was performed with illumination from a high-pressure mercury lamp.

The MICs of the four macrolide antibiotics assayed by the agar method are shown in Fig. 1A. EM (MIC for 90% of isolates [MIC₉₀], 0.006 μg/ml), RKM (MIC₉₀, 0.015 μg/ml), JM (MIC₉₀, ≥0.03 μg/ml), and LM (MIC₉₀, ≥0.03 μg/ml) were similarly active against the organism.

The minimum doses of antibiotics to eliminate M. pneumoniae from L cells are shown in Fig. 1B. RKM was the most effective for the elimination of M. pneumoniae from L cells. More than 90% of strains were completely eliminated in 7 days of cultivation with 0.007 μg of RKM per ml. Similarly, the doses of EM, JM, and LM to eliminate 90% of strains were 0.03, 0.03, and ≥0.06 μg/ml, respectively. We

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FIG. 1. Susceptibilities of 50 strains of *M. pneumoniae* to macrolide antibiotics assayed by the agar dilution method (A) and in mycoplasma-infected L cells (B). Symbols: ■, EM; ●, RKM; △, JM; and ▼, LM. The numbers of strains are indicated at the bottom (F, strain FH).

cleared whether the cell cultures were "cured" by using DNA staining. As shown in Fig. 2, the microorganisms had completely disappeared 3 days after treatment with RKM (1 µg/ml), and the cells looked healthy. The cured L cells were continuously cultured for more than 1 month without the antibiotics, and no mycoplasmas were found.

The MICs determined by the agar dilution method for individual strains were compared with the minimum doses needed to eliminate the microorganisms from L cells (Fig. 3). The results showed that the doses of RKM for the elimination of *M. pneumoniae* from L cells were lower than the MICs in 41 strains and higher in 6 strains. Those of JM and LM were almost proportional to their MICs. However, it is interesting that relatively high doses of EM were required for the elimination of mycoplasmas (more than 80% of strains).

We demonstrated the susceptibilities of *M. pneumoniae* in L cells to macrolide antibiotics. The assay system used to determine the activities of antibiotics in this study might be clinically useful for the treatment of infections caused by *M. pneumoniae*, because attachment of viable mycoplasmas to the respiratory epithelium is necessary for the initiation of infection (2, 7, 9). The mechanisms of attachment of *M. pneumoniae* to L cells are not clear, but the attachment might be due to a sialic acid-containing region, in a manner similar to that of the attachment of *M. pneumoniae* to the respiratory epithelium and erythrocytes (2, 6).

It is well known that macrolide antibiotics, especially EM, are very active against *M. pneumoniae* in vitro (1, 10). However, we demonstrated that doses of EM required to eliminate most *M. pneumoniae* strains from L cells were higher than doses of RKM, JM, and LM. The reason is not clear, but RKM and the others might be carried more easily through intracellular space than is EM.

To clarify correlations between the effects of antibiotics on the elimination of mycoplasmas from infected cells and the clinical effects of these antibiotics on mycoplasma infections, further studies, particularly comparisons of the clinical effects of EM and RKM, are needed.

FIG. 2. L cells infected with *M. pneumoniae*. (A) Fluorescence pattern is observed in cell nuclei and mycoplasmas by DNA staining. (B) L cells cured by 1 µg of RKM per ml. No mycoplasma cells, i.e., only brightly fluorescing cell nuclei, are seen.
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