Usefulness of an Erythromycin-Resistant Strain of *Mycoplasma pneumoniae* for the Fermentation-Inhibition Test

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The fermentation-inhibition (FI) test for *Mycoplasma pneumoniae* was performed with two strains of *M. pneumoniae*, one susceptible to erythromycin and one highly resistant to erythromycin with cross-resistance to other macrolide antibiotics. Serum titers in children with *M. pneumoniae* pneumonia who received no antibiotic were similar with the two strains. Children with atypical pneumonia with a transient rise in the FI titer with the susceptible strain proved to have received erythromycin at the time of the rise. They showed no rise in the FI titer done with the erythromycin-resistant strain. Oral administration of erythromycin regularly elevated the serum FI titer when the test was done with the susceptible strain. Use of the resistant strain in the test eliminated this false elevation. Tetracycline and chloramphenicol did not elevate the titer, even with the susceptible strain. Use of the strain of *M. pneumoniae* resistant to erythromycin provides a true FI antibody serum titer, avoiding the influence of antibiotics.

Two methods of metabolic inhibition testing were devised for the measurement of serum antibody to *M. pneumoniae*. The tetrazolium reduction-inhibition test was reported by Jensen (K. E. Jensen, Bacteriol. Proc., p. 70-71, 1964) and Senterfit and Jensen (2). The fermentation-inhibition (FI) test devised by Taylor-Robinson et al. (4) is especially useful in sero-diagnostic and seroepidemiological studies of *M. pneumoniae* infections. However, Mogabgab (1) reported that the administration of antibiotics, especially erythromycin, elevated serum tetrazolium reduction-inhibiting titers. Smith and Herrmann (3) reported that there appeared to be no practical way whereby this antibiotic interference in the metabolic inhibition test could be eliminated.

We succeeded in making *M. pneumoniae* highly resistant to erythromycin in vitro (Y. Niitu et al., Antimicrob. Ag. Chemother., submitted for publication). Performance of the FI test with both the erythromycin-sensitive and -resistant strains of *M. pneumoniae* revealed the usefulness of the resistant strain in avoiding false results in sera from patients receiving certain antibiotics.

MATERIALS AND METHODS

M. pneumoniae. The Mac strain, a prototype *M. pneumoniae*, was used as the erythromycin-sensitive strain. The Mac strain was rendered resistant to erythromycin. The minimal growth-inhibitory concentration by serial subculture in broth media containing erythromycin. The minimum growth-inhibitory concentrations (MGIC) of antibiotics for these strains are shown in Table 1. The two strains were propagated in broth medium, distributed into tubes, and kept at

-70 C. The two cultures contained 1×10^6 colony-forming units per ml.

Media. Broth medium for the FI test consisted of seven parts of pleuropneumonia-like organism broth (Difco), two parts of unheated horse serum, and one part of 25% yeast extract. In addition, 1% glucose, 500 U of penicillin G per ml, 500 μ g of thallium acetate per ml, and 0.002% phenol red were included. The pH was adjusted to 7.8.

Fermentation-inhibition test. The details of the method which we used (4) have been described elsewhere (S. Hasegawa, Jap. J. Microbiol., in press). Sera were stored at -20 C and were heated at 56 C for 30 min before use. Serial twofold dilutions of serum were prepared, beginning with a dilution of 1:4. Broth containing *M. pneumoniae* was diluted 10^{-3} with broth medium for use as inoculum. Plates were sealed with cellophane tape, and the center of the tape over each cup was pierced with a needle to prevent nonspecific color change of the medium to slightly yellow. The titer was recorded when the control cup with only *M. pneumoniae* changed color to yellow (pH 6.7 or less). The highest initial serum dilution which prevented the distinct color change, when the pH of the medium was kept at 7.4 or over, was considered the antibody titer of the serum.

Source of sera. The FI antibody titer with the sensitive and resistant strains was determined on paired or serial sera from six children. These children were diagnosed as having *M. pneumoniae* pneumonia by isolation of *M. pneumoniae* or by a significant rise in complement-fixing antibody titer (or both) and were not treated with antibiotics.

Erythromycin, josamycin, leucomycin, oleandomycin, spiramycin, lincomycin, tetracycline, and chloramphenicol were given orally to three to five subjects (adults and children, respectively) in a single dose of about 7 to 9 mg/kg of body weight (one-fourth of the daily dose). Serum was drawn before and 2, 4, 6, and 12 h after administration of drugs. The FI titer of

TABLE 1. Antibiotic susceptibility of the erythromycin-sensitive and -resistant strains of *M. pneumoniae*

Antibiotic	Minimal growth inhibitory concentration ($\mu\text{g/ml}$)	
	Erythromycin-sensitive strain	Erythromycin-resistant strain
Erythromycin	0.016	800
Josamycin	0.032	10
Leucomycin	0.064	10
Oleandomycin	0.2	1,600
Spiramycin	0.4	40
Lincomycin	12.8	200
Tetracycline	0.2	0.2
Chloramphenicol	12.8	6.4
Streptomycin	40	40

these sera was determined with the sensitive and resistant strains.

RESULTS

FI antibody titers. FI antibody titers were determined with erythromycin-sensitive and -resistant strains in children with *M. pneumoniae* pneumonia. The antibody titers and the titer rises in the six children with *M. pneumoniae* pneumonia, as determined with the resistant strain, were not significantly different from those done with the sensitive strain, which hitherto has been used in the FI test (Fig. 1).

Analysis of cases with a transient rise in fermentation-inhibition titer. Mass chest X-ray surveys for the detection of pulmonary tuberculosis were done among school children in Sendai in 1968 and 1969. One hundred and forty-four children with atypical pneumonia had sera drawn three to five times—at the first examination, about 3 weeks later, and in-between. *M. pneumoniae* infections were precluded in these children by a negative culture for *M. pneumoniae* and a low complement-fixing antibody titer (1:32 or below) to *M. pneumoniae* which remained stable.

In 30 of the 144 cases there was a transient rise in FI titer when the test was performed with the erythromycin-sensitive strain (Table 2). The titer of the first serum was below 1:4 in 26 cases and was 1:4 in four cases. The titer of the last serum obtained was not significantly different from the titer of the first serum in any of the 30 cases. However, 37 of the 57 in-between examination sera showed a significant rise in FI titer as compared with the first examination sera. Of the 37 in-between examination sera, the titer was 1:8 in 11, 1:16 in 9, 1:32 in 10, 1:64 in 4, 1:128 in 2, and 1:256 or over in 1. If only the first and in-between examination sera had been obtained, all 30 cases might have been erroneously diagnosed as *M. pneumoniae* infections.

The transient rise in FI titer could be caused

FIG. 1. Comparison between fermentation-inhibiting antibody titers with erythromycin-sensitive and -resistant strains in serial sera drawn from children with *M. pneumoniae* pneumonia.

TABLE 2. Serum FI titers with erythromycin-sensitive and -resistant strains in three representative children with atypical pneumonia in whom *M. pneumoniae* infection was precluded

Case no.	Date	FI titer ^a	
		S	R
1	May 17	<4	<4
	25 ^b	64	<4
2	June 7	<4	<4
	June 3	<4	<4
	7 ^b	32	<4
	13	<4	<4
3	May 17	<4	<4
	24 ^b	8	<4
	31 ^b	32	<4
	June 12	<4	<4

^a S, Erythromycin-sensitive strain; R, erythromycin-resistant strain.

^b The day erythromycin was taken by the patients.

by an antibiotic in the serum. Twenty-nine of the 30 cases proved to have been receiving erythromycin on the day the 37 in-between examination sera with transient rise in titer were drawn and no antibiotic on the day the first and last examination sera were drawn. In one case it was not clear whether any antibiotic had been given.

All serial sera from the 30 cases were tested

with the resistant strain. As with the sensitive strain, the titer of the first examination serum was below 1:4 in 29 cases, 1:4 in one case, and was not significantly different from the titer of the last examination serum. The in-between examination sera were not significantly different in titer from the first examination sera, showing no transient rise in FI titer in any of the 30 cases.

Seven additional cases were receiving tetracycline and one case was receiving chloramphenicol on the day the in-between examination serum was drawn. None of these cases showed any significant rise in FI titer as determined with the sensitive strain.

Effect of oral administration of antibiotics on the FI titer of serum. All four individuals who received erythromycin, two of five who received leucomycin, and one of five who received oleandomycin showed a titer rise after drug administration when a susceptible strain was used in the FI test. However, no significant change in the titer was detected when the erythromycin-resistant strain was employed in the test (Fig. 2). None of the other three and four individuals who received leucomycin and oleandomycin, respectively, and none of all

three individuals who received josamycin, lincomycin, tetracycline, or chloramphenicol showed a significant change in titer, regardless of whether erythromycin-sensitive or -resistant strains were used in the test.

DISCUSSION

When the FI test was performed with an erythromycin-sensitive strain of *M. pneumoniae*, the oral administration of erythromycin caused a rise in serum titer. A similar rise was seen in some cases after oral administration of leucomycin or oleandomycin, but not with josamycin, spiramycin, and lincomycin. When the erythromycin-resistant strain was used in the test, the false rise in titer was not observed. An antibiotic with a low MGIC is likely to cause such a false rise. The MGIC for the erythromycin-sensitive strain was the lowest for erythromycin and relatively low for leucomycin and oleandomycin among the antibiotics studied (Table 1). The fact that josamycin, which has a relatively low MGIC, did not elevate FI titer after being orally administered suggests that its serum concentration did not reach a high level.

Tetracycline and chloramphenicol, to which both the erythromycin-sensitive and -resistant strains were sensitive, did not cause a false rise in the FI titer after being orally administered. The reason may be that the MGIC of the two antibiotics were higher than those of erythromycin, as shown in Table 1, and that their serum concentration did not exceed the MGIC enough to affect the FI titer.

The test with the erythromycin-resistant strain can determine the true FI antibody titer in sera drawn from patients receiving erythromycin and certain other antibiotics or collected from a population for seroepidemiological study. The use of this strain may give true results in serodiagnosis of *M. pneumoniae* infections, avoiding the possible interference by the antibiotics. It is also assumed that the strain might avoid false results in the tetrazolium reduction-inhibition test in sera drawn after the antibiotics are given.

It may be concluded that the strain of *M. pneumoniae* resistant to erythromycin is suitable for the metabolic inhibition test for the measurement of antibody to *M. pneumoniae*.

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FIG. 2. Comparison of FI titers in sera of seven individuals after a single oral dose of antibiotic with