EFFECT OF ANTIBIOTICS ON MYCOPLASMA*

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(Received for publication July 11, 1970)

When using tissue culture as a tool for screening antitumor agents, most cell cultures were contaminated with mycoplasma and attempts were made to eliminate the mycoplasma from the cell cultures with antibiotics. Several strains of mycoplasma tested were highly sensitive to macrolide antibiotics and antitumor antibiotics. Among macrolide antibiotics, leucomycin group such as leucomycin, spiramycin and tylosin were more active than erythromycin group, such as erythromycin and oleandomycin. In contrast to the results *in vitro*, mycoplasma infected naturally in the cultured cells was highly resistant to the antibiotics, but mycoplasma (strain Campo) inoculated in embryonated eggs was completely eliminated with 2 injections of 40 mcg/egg of leucomycin.

Tissue culture is often used as a tool for screening antitumor agents, but many reports have been made of tissue culture contamination by mycoplasma, since ROBINSON *et al.*[1] first described it in 1956. On the other hand, NEWNHAM *et al.*[2] reported that strains of mycoplasmas were sensitive to several known antibiotics *in vitro*. The present authors have attempted to eliminate mycoplasma from experimantal animals and cell cultures with antibiotics, especially with leucomycin.

Materials and Methods

Strains of Mycoplasma

Mycoplasmas used in this study were the following 5 strains: Strains 07 (*Mycoplasma hominis* type 2), A-63 (unidentified), and Campo (*M. hominis* type 2) were isolated from human sources, strain KP-13 (*M. gallisepticum*) from a chicken, and strain 15T (unidentified) from a rat. All were obtained from the Department of Microbiology, Keio University Medical School.

Antibiotics Tested

The antibiotics used were as follows: Broad spectrum antibiotics such as chloramphenicol, tetracycline, and chlortetracycline; macrolide antibiotics such as carbomycin, erythromycin, oleandomycin, leucomycin A_1, spiramycin, and tylosin; antitumor antibiotics such as actinomycin S, mitomycin C, G-253, chromomycin A_3, pactamycin, puromycin, streptonigrin, nucleocidin, and quinomycin A; antibacterial antibiotics such as cycloserine, fradiomycin (neomycin), kanamycin, streptomycin, polymyxin B, chartreusin, and helvolic acid; antifungal antibiotics such as amphotericin B, fumagillin, nystatin, oligomycin, trichomycin, and cerulenin.

* Suppported in part by grant CA-05085 from National Cancer Institute, Public Health Service, U. S. A.

A preliminary report was presented at the 39th Annual Meeting of the Japanese Society of Bacteriology in 1966[3].

Culture Medium Used

Though various culture media were used for the detection of mycoplasmas, a new mycoplasma medium "Hokken Mycoplasma Agar or Broth" was employed in the experiments. This medium consists of 0.7 % Hokken heart extract, 1.0 % purified peptone, 0.1 % yeast extract, and 0.5 % sodium chloride. It was used with or without 0.8 % purified agar at pH 7.8. After autoclaving for 15 miuntes at 121°C, it was cooled to 50~55°C, and added with 15 ml of mycoplasma serum from which a growth inhibiting factor for mycoplasma was eliminated by a specific method. It was found that the growth of mycoplasma on this medium was superior to those used hitherto. Mycoplasma suspension of 10^5~10^8 viable units/ml were used by diluting with the mycoplasma broth.

Detection of Mycoplasmas

For the detection of mycoplasmas, a small portion (0.02 ml) of the test solution was placed in agar plates prepared with the mycoplasma medium. Very small mycoplasma colonies became visible on the agar plate after incubation for 4 days at 37°C. Growth was confirmed under a microscope at a low magnification (×60). All the strains used in this experiment grew well on this medium.

The colonies of these mycoplasmas grown on the mycoplasma agar plate after incubation for 96 hours were generally multiform, and varied in size and external appearance according to the strains used. Strains Campo and 15T showed a "fried egg" appearance with a convex center. The convex center became recognizable after 48-hour incubation.

Experimental Results

1. *In vitro* Sentitivity of Mycoplasmas to Various Antibiotics

Agar plates were prepared by adding various antibiotics to the mycoplasma agar medium in various concentrations. The test strain grown in the mycoplsma broth was inoculated onto the plates. After incubation for 96 hours at 37°C the minimal inhibitory concentration was determined as indicated in Table 1. All the strains used in this experiment were sensitive to the macrolide antibiotics, leucomycin, spiramycin, tylosin and carbomycin, and to the antitumor antibiotics, actinomycin S, chro-

Table 1. Effect of antibiotics on 6 strains of mycoplasmas *in vitro*

Antibiotic	Strains and MIC of antibiotics (μg/ml)				
	15T	KP-13	07	Campo	A-63
Cycloserine	50	—	100	—	—
Fradiomycin	25	12.5	6.3	3.1	12.5
Kanamycin	100	6.3	6.3	0.4	1.6
Streptomycin	100	100	100	100	100
Chloramphenicol	3.2	0.4	12.5	3.2	1.6
Chlortetracycline	6.3	0.4	5	1.2	0.4
Tetracycline	6.3	1.6	1.6	1.6	1.6
Erythromycin	12.5	12.5	12.5	25	12.5
Oleandomycin	100	12.5	25	12.5	12.5
Carbomycin	12.5	3.1	3.1	3.1	3.1
Leucomycin	1.6	0.8	1.6	0.4	0.8
Spiramycin	0.8	0.4	6.25	3.1	0.8
Tylosin	0.8	1.6	1.6	6.3	6.3
Amphotericin B	25	12.5	50	12.5	—
Oligomycin	6.3	50	50	50	—
Trichomycin	6.3	12.5	12.5	12.5	6.3
Nystatin	100	12.5	100	50	100
Fumagillin	50	50	50	25	—
Cerulenin	100	12.5	100	100	50
Actinomycin S	0.4	0.4	0.4	0.4	0.4
Chromomycin A_3	0.8	0.15	1.5	6.15	0.65
Mitomycin C	0.01	0.16	0.16	0.16	0.12
G-253 C	10	0.16	0.16	1.6	0.06
Puromycin	1.6	0.1	0.4	0.8	12.5
Quinomycin A	0.4	0.16	1.6	0.8	0.8
Streptonigrin	50	12.1	3.1	3.1	6.3
Nucleocidin	12.5	6.3	25	6.3	12.5
Carzinostatin	25	25	50	25	—
Pactamycin	100	100	100	100	100
Chartreusin	50	25	6.3	6.3	12.5
Helvolic acid	100	100	100	100	100
Polymyxin B	100	50	100	50	50

momycin A_3, mitomycin C, puromycin, and quinomycin. The activity of erythromycin and oleandomycin was weaker than the other macrolides. Chloramphenicol, tetracycline, and chlortetracycline were effective. Of the basic glucoside antibiotics, streptomycin had almost no activity. There were no effective antifungal antibiotics, except for trichomycin, which was slightly effective.

2. Elimination of Naturally Infected Mycoplasmas from Tissue Cell Lines

When the suspensions of tissue cell lines of L, FL, HeLa, and KB preserved in our laboratory were inoculated on the mycoplasma agar medium, mycoplasma was detected except in the KB cells.

For the purpose of eliminating mycoplasma from these cultured cells, 10^5 cells/ml of HeLa cells contaminated with mycoplasma were incubated in HANK's balanced salt solution (HSS), which contained 100 units/ml of penicillin and 100 mcg/ml of streptomycin, at 37°C for 2 days. The culture was then transferred to HSS containing various amounts of antibiotics, and incubated for 10 days. Examination for mycoplasma was made at intervals. Leucomycin A_1 and G-253 (an antitumor antibiotic similar to mitomycin) were used. Concentrations of 5~100 mcg/ml of leucomycin A_1, 0.5 mcg/ml of G-253, and 100 units of penicillin plus 100 mcg/ml of streptomycin had no influence on the growth of the cultured cells.

Table 2. Effect of antibiotics on mycoplasma in HeLa cells

Concentration (μg/ml)				Total number of mycoplasma colonies/ml in the culture				
					After			
Leucomycin A_1	Penicillin	Strepto-mycin	G-253	Before treatment	2 days	5 days	7 days	10 days
100				10*~60	1~2	0	2~5	10
50				10~60	1~2	0	2~5	10
20				10~60	2~5	1~2	2~5	10~60
10				10~60	2~5	1~2	2~5	10~60
5				10~60	1~2	2~5	10	10~60
100			0.5	10~60	10~60	0	1~2	1~2
50			0.5	10~60	1~60	0	1~2	2~5
			0.5	10~60	10~60	1~2	1	10~60
20	100	100		10~60	2~5	0	10	10~60
10	100	100		10~60	2~5	2~5	2~5	10~60
5	100	100		10~60	2~5	2~5	10~60	10~60
Control				10~60	>60	>60	>60	>60

* Number of colonies grown on mycoplasma agar plate.

As shown in Table 2, total number of mycoplasma in the broth culture was decreased 2 days after addition of leucomycin A_1 alone or with G-253. After 5 days with 50 and 100 mcg/ml of leucomycin alone or with G-253, no colonies were observed, but after 7 days a few colonies reappeared. With 20~5 mcg/ml of leucomycin A_1 the number of mycoplasma was decreased after 5 days, but was the same as before treatment after 10 days.

3. Antibiotic Sensitivity to Mycoplasma Infected in KB Cells

KB cells were used in the following experiment because they were found to be

naturally free from mycoplasma. Mycoplasma strain 15T, A-63, or 07 was inoculated into the KB cell culture tubes, incubated for 3 days, and then antibiotics which were effective against these organisms *in vitro* were added at various concentrations and incubated at 37°C for 4 days.

As shwon in Table 3, mycoplasmas in KB cells were remarkably resistant to the antibiotics compared to those mycoplasmas free from tissue cells.

Table 3. Effect of antibiotics against 3 strains of mycoplasma tested in the KB cell culture

Antibiotic	MIC of antibiotics (μg/ml)		
	A-63	15T	07
Chloamphenicol	250	500	500
Tetracycline	50	250	50
Chlortetracycline	100	250	250
Erythromycin	250	250	100
Oleandomycin	500	1,000	500
Leucomycin	250	250	100
Spiramycin	250	250	500
Chartreusin	500	500	500
Mitomycin C	3.2	1.6	3.2

4. Experimental Treatment of Mycoplasma-infected Mice with Antibiotics

Female mice (*ddN*), weighing 20 g, were obtained from an animal breeder and the mouth and genital organ of the mice were washed with physiological saline solution. Mycoplasma was positive in 22 % of the washings from throat, in 20 % from larynx, 12 % from trachea, and in 32 % from genitalia. These mice were infected intranasally with 0.02 ml of a broth culture of Campo strain containing 10^6~10^8 cells/ml. By this artificial infection, the number of mycoplasma colonies was increased. A single intraperitoneal injection of 400 mg/kg of leucomycin A_1 or kanamycin was made in 5 mice each. Mice were sacrificed 3 days after the treatment. Organs were removed and washed with about 10 volumes of physiological saline solution. The washings were examined for mycoplasma. As shown in Table 4, in the group of mice treated with leucomycin A_1, the number of mycoplasma in mouth and trachea decreased to one-fourth and one-half, respectively, while in the group treated with kanamycin, the number of mycoplasmas did not decrease as much.

Table 4. Isolation of mycoplasma from mice treated with antibiotics

	Average number of mycoplasma colonies detected in 0.02 ml of washings			
	Mouth	Larynx	Trachea	Genital organ
Untreated control	39	32	16.9	53.5
Treated with leucomycin (400 mg/kg)	9.5	26.2	9	32
Treated with kanamycin (400 mg/kg)	19	29.5	25.6	33.2

5. Experimental Treatment of Mycoplasma in Embryonated Chicken Eggs with Antibiotics

Aliquots (0.2 ml) of a suspension containing 10^8 cells of strain Campo per ml were injected into the yolk of 8-day-old hatching egg. Each group consisted of three eggs. Twenty four hours after the inoculation, various amounts of leucomycin A_1, spiramycin, tylosin, kanamycin, or tetracycline were injected once into the yolk of the embryonated eggs. After 24, 48, and 96 hours, 0.02 ml of allantoic fluid was withdrawn and incubated on the mycoplasma agar plate. The results in Table 5 show that all the antibiotics tested completely prevented the proliferation of mycoplasma at a dose of 2 mg/egg. With a dose of 400 mcg/egg leucomycin A_1, kanamycin, and

Table 5. Effect of antibiotics on mycoplasma infection in embryonated chicken eggs

Antibiotic	Administration	Number of eggs in which mycoplasma was found														
		2,000 μg/egg			400 μg/egg			40 μg/egg			4 μg/egg			Control		
		24 hrs.	48 hrs.	96 hrs.	24 hrs.	48 hrs.	96 hrs.	24 hrs.	48 hrs.	96 hrs.	24 hrs.	48 hrs.	96 hrs.	24 hrs.	48 hrs.	96 hrs.
Leucomycin	single	0	0	0	0	0	0	1	1	2	1	3	2	3	3	3
	2 times	0	0	0	1	0	0	0	0	0	2	3	3	3	3	3
	3 times	0	0	0	0	0	0	0	0	0	3	2	3	3	3	3
Tylosin	single	0	0	0	0	0	0	0	2	3	3	3	3	3	3	3
Spiramycin	single	0	0	0	1	2	3	3	2	3	3	3	3	3	3	3
Kanamycin	single	0	0	0	0	0	0	1	2	3	3	3	3	3	3	3
Tetracycline	single	0	0	0	3	3	3	3	3	3	3	3	3	3	3	3

* Remarks: Treatment started 24 hours after challenge of *Mycoplasma* strain Campo.

tylosin eliminated the mycoplasma completely, but the organism remained positive with tetracycline and spiramycin. With 40 mcg/egg of leucomycin A_1, no organism was found after it was administered once a day for 2 days.

Discussion

The mode of activity of antibiotics against various mycoplasmas is different from that against bacteria. Though mycoplasmas are highly sensitive to the macrolide antibiotics, they are less sensitive to erythromycin and oleandomycin and are highly sensitive to leucomycin, spiramycin and tylosin. Leucomycin[4,5], spiramycin[6] and tylosin[7] have a aldehyde functional group on the lactone ring, but erythromycin and oleandomycin do not. MITSUHASHI *et al.*[8] pointed out in a study on the resistance of *Staphylococcus* to the macrolide antibiotics that C type of this organism is affected differently by erythromycin or oleandomycin than by leucomycin or spiramycin. The former two antibiotics can induce macrolide resistance, but the latter two cannot.

Some of the antitumor antibiotics show high activity against mycoplasmas, suggesting that inhibitors of nucleic acid biosynthesis are active against mycoplasmas. However, since the antitumor agents used in this experiment are toxic, these antibiotics probably cannot be used therapeutically against mycoplasma infection.

The *in vitro* susceptibility of mycoplasmas to various antibiotics has been reported by several researchers[9~13]. Macrolide antibiotics showed a high activity against mycoplasmas of human or animal origin, with minimum inhibitory concentrations of these antibiotics lower than those described in this paper. According to NIKAIDO[12], leucomycin was most effective in that a 2 mcg/ml concentration was enough to eliminate *M. hominis* infected artificially in L cell cultures. Campo strain (*M. hominis* type 2) infected in KB cells was not completely eliminated from KB cell cultures with 5~10 mcg/ml concentration of leucomycin in our experiments. The discrepancy between these results may be caused by assay media used.

Acknowlegements

We are grateful to Prof. SASAKI of the Keio University Medical School for kindly providing us with the test strains of mycoplasmas, to Dr. YOSHIOKA of this Institute for kindly providing us with his mycoplasma media and to Dr. IGARASHI of this Institute for his kind help with embryonated eggs.

We are also indebted to CCNSC, National Cancer Institute, NIH, Bethesda, U. S. A. for supplying some of the antibiotics used.

References

1) Robinson, L. B.; R. H. Wichelhausen & B. Roizman : Contamination of human cell cultures by pleuropneumonia-like organisms. Science 124 : 1147, 1956
2) Newnham, A. G. & H. P. Chu : An *in vitro* comparison of the effect of some antibacterial, antifungal and antiprotozoal agents on various strains of mycoplasma. J. Hyg. Camb. 63 : 1~23, 1965
3) Hoshino, Y.; T. Maekawa, I. Umezawa, H. Yamamoto, A. Matsumae & T. Hata : Studies on leucomycin; Effect of leucomycin on mycoplasma (PPLO). Proc. 39th Annual Meeting of Japan Soc. Bacteriol., p. 86, 1966.
4) Ōmura, S.; M. Katagiri & T. Hata : The chemistry of leucomycin. IV. Structure of leucomycin A_1. J. Antibiotics 21 : 199~203, 1968
5) Ōmura, S.; M. Katagiri & T. Hata : The chemistry of leucomycin. VI. Structure of leucomycin A_4, A_5, A_6, A_7, A_8 and A_9. J. Antibiotics 21 : 272~276, 1968
6) Ōmura, S.; A. Nakagawa, M. Ohtani, T. Hata, H. Ogura & K. Furuhata : Structure of spiramycins and their relationship with the leucomycins and carbomycins. J. Amer. Chem. Soc. 91 : 3401~3404, 1969
7) Morin, R. B. & M. Gorman : The partial structure of tylosin, a macrolide antibiotic. Tetrahedron Letter 34 : 2339~2345, 1964
8) Kono, M.; H. Hashimoto & S. Mitsuhashi : Drug resistance of staphylococci. III. Resistance to some macrolide antibiotics and inducible system. Jap. J. Microbiol. 10 : 59~66, 1966
9) Arai, S.; Y. Yoshida, A. Izawa, K. Kumagai & N. Ishida : Effect of antibiotics on growth of *Mycoplasma pneumoniae* Mac. J. Antibiotics, Ser. A 19 : 118~120, 1966
10) Ōmura, S.; Yong-Chi Lin, T. Yajima, S. Nakamura, N. Tanaka, H. Umezawa, S. Yokoyama, Y. Homma & M. Hamada : Screening of antimycoplasma antibiotics. J. Antibiotics, Ser. A 20 : 241~245, 1967
11) Arai, S.; K. Yuri, A. Kudo, M. Kikuchi, K. Kumagai & N. Ishida : Effect of antibiotics on the growth of various strains of mycoplasma. J. Antibiotics, Ser. A 20 : 246~253, 1967
12) Nikaido, H. & K. Katagiri : *In vitro* susceptibility of mycoplasmas to antibiotics. Proc. Jap. J. Bact. 21 : 486, 1966