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A Method for Eradication of *Mycoplasma* Infections in Cell Cultures (29735)

GIO BATTA GORI AND DOO YOUNG LEE (Introduced by H. Eagle)
Microbiological Associates, Inc., Bethesda, Md.

Investigators using cell cultures have become increasingly aware of the problems introduced by parasites such as *Mycoplasma*.

In the past few years many have attempted to eliminate *Mycoplasma* in tissue cultures, using different methods and with varying degrees of success. Heat treatment (Hayflick (7)), use of specific antiserum (Pollock and Kenny(2)) or antibiotic treatment (Hearn, Officer, Elsner, and Brown(3); Pollock, Kenny and Syverton(4); Koehler(5)) have all been suggested. In our hands these methods have not been consistent, in that they may have been successful only in particular cell lines and with particular strains of *Mycoplasma* contaminants, or on account of more sensitive methods (Hayflick(1)) used in our laboratory to isolate aerophilic and microaerophilic *Mycoplasma* (Table I). As the morphology of isolates suggested that a variety of *Mycoplasma* was infecting the screened cell lines, the preparation of polyvalent antisera was not considered practical, and it was decided instead to explore fully the usefulness of antibiotic treatment. Simple antibiotic treatment, even at near cytotoxic doses and for periods of up to 5 months, proved only capable of depressing the severity of infection, approximately from 100 or more to 10 or less colonies per million cells, without effecting sterilization (Table I). One obvious difficulty was the fact that *Mycoplasma* infection can be and usually is an intracellular infection (Edwards and Fogh (6)); Hayflick and Stinebring(7); Carski and Shepard(8)). In consequence, antibiotics or specific antisera may have eliminated the *Mycoplasma* present in culture fluids without affecting those nested inside cells. A possible approach was to increase the permeability of the cells and, of the several possible methods, a simple hypotonic treatment was selected.

Materials and methods. Antibiotic-Hypotonic treatment. Previous studies (Blyth(9); Hearn, Officer, Elsner, and Brown(3); Fogh and Hacker(10); Pollock, Kenny and Syverton(4); Koehler(5)) indicate that *Mycoplasma* are highly sensitive to aureomycin,

TABLE I. *Mycoplasma* Infection in Heteroploid Cell Cultures Before and After Antibiotic Treatment.

Cell line	Before treatment A	Before treatment M	Antibiotic treatment A	Antibiotic treatment M
D-6, a (Berman)	+	+	—	+
D-98, a "	+	+	+	+
Conjunctiva, a (Chang)	+	+	—	+
FL-Amnion, a (Fogh)	—	+	—	+
L-929, a (Sanford)	—	+	—	+
HeLa-S3, a (Puck)	—	+	—	+
HeLa-S3-1, a (Saltzman)	—	+	—	+
HeLa, a (Gey)	—	+	—	—
HeLa, b "	+	+	+	+
AV-3, a (Robbins)	—	+	—	+
WISH, a (Hayflick)	—	+	—	+
KB, a (Eagle)	+	+	—	+
KB, c "	—	—		
KB, b "	+	+	—	+
Monkey heart, a (Salk)	+	+	—	+
J-111, a (Osgood)	+	+	—	+
G-99, a (Girardi)	+	+	—	+
S-91, a (Foley)	—	+	—	+
CA-755, a (Girardi)	+	+	—	+
S-180, a (Foley)	—	+	—	+
Human heart, a (Girardi)	—	+	—	+
Liver, a (Chang)	+	+	+	+

A, aerobic; M, microaerophilic.
a, calf serum cultivated; b, horse serum cultivated; c, human serum cultivated.

kanamycin, and chloroamphenicol. Suspensions or monolayers of *Mycoplasma* infected cell cultures were exposed for 2-5 minutes to distilled water containing these antibiotics in combination at cytotoxic levels, namely 1,000 μg/ml of aureomycin, 10,000 μg/ml of kanamycin and 250 μg/ml of chloroamphenicol. Cells so treated were then placed in stationary flasks in Basal Medium Eagle with 10% calf serum and antibiotics at one-fifth the concentration previously provided in water. Extensive damage of the cells was observed, with survival and attachment of 30-50% of the original cell population. The medium was changed 2 or 3 times at 2-3 day intervals, and then substituted with Basal Medium Eagle with 10% calf serum and 5 μg of aureomycin per ml. In this last medium the cells were propagated indefinitely.

Mycoplasma isolation. The agar medium for *Mycoplasma* isolation was described by Hayflick(1) and used successfully by Chanock, Mufson, James, Fox, Bloom and Forsyth (11). Isolation was performed both aerobically and in microaerophilic environment (atmosphere changed 3 times with 5% carbon dioxide in nitrogen) and 37°C and 100% humidity.

Bacteriological plastic dishes, 60 mm diameter, were used through the experiments. Colonies were counted at 200 power with an inverted microscope 4 to 6 days after inoculation. For each experiment a medium control was performed by inoculating a *Mycoplasma* positive specimen. Every second week a culture of each cell line treated for *Mycoplasma* elimination was propagated and fed at least twice in antibiotic-free medium. It was then harvested, suspended at 5 million cells per ml, frozen and thawed once to disrupt the cells, and distributed in .2 ml aliquots in 6 dishes of *Mycoplasma* agar: 3 to be incubated aerobically and 3 in microaerophilic environment. If a positive result was obtained, the cell culture was again subject to the hypotonic-antibiotic treatment.

Virus titrations. Virus dilutions of .5 $\log_{10}$ steps were inoculated in .5 ml amounts per culture, 10 cultures per dilution. Polio virus type 1 (Mahoney strain) titrations were evaluated in 5 days. Measles virus (Edmonston strain) titrations were evaluated in 9-10 days. Vaccinia virus (CAM-15 strain) titrations were evaluated in 5-6 days. Titers were calculated by the Kärber equation and are expressed as $\log_{10}$ of $TCID_{50}$/ml.

Experimental results. Primary cell cultures were usually found to be *Mycoplasma* free, except for occasional cultures of human embryonic skin, human embryonic lung or amnion. A number of diploid cell cultures were tested for *Mycoplasma*, and in a few instances were found with low degrees of infection, as indicated in Table II. The hypotonic-antibiotic treatment proved to be quite lethal for diploid cells, to the point that only a few cells recovered. The difficulty of growing diploid cells with extremely small inocula makes this method quite impractical for diploid cell cultures.

The best results were experienced with heteroploid cell cultures. A survey for *Mycoplasma* contamination among some heteroploid lines carried by this laboratory is summarized in Table I. Twelve of these lines,

TABLE II. *Mycoplasma* Infection in Diploid Cell Cultures.

Cell line	A	M
Bovine lung	—	—
Bovine conjunctiva	—	+
MAF, human embryonic skin & muscle	—	+
M-7, " " lung	—	—
M-5, " " skin	—	—
WI-26, " " lung (Hayflick)	+?*	+
WI-38, " " " "	—	—
MA-10, " " kidney	—	+
MA-11, Patas monkey kidney	—	—
BSC-1, African green monkey kidney† (Hopps)	—	—
Rabbit skin, adult	—	—
Human embryonic buccal mucosa	—	—
" " conjunctiva	—	—
" " trachea	—	—

A, aerobic; M, microaerophilic.
* Probable contamination. † Subdiploid.
Note: All lines, unless otherwise referred, were developed by Microbiological Associates, Inc.

previously *Mycoplasma* contaminated, have been made *Mycoplasma* free by the method described and are still free 14 months after treatment (Table III).

Comparative virus titrations before and after antibiotic-hypotonic treatment did not indicate significant variations of the virus sensitivity of the treated lines (Table IV). In the case of heavily contaminated lines it rather appeared that this sensitivity is improved after treatment. A similar observation was made by Dr. J. Sever (NIH, Bethesda, Md.) growing Rubella virus in RK-13 cells (Dudgeon) made *Mycoplasma* free by the method described.

Discussion. Cultured cells are definitely subjected to selection by the method described. However, it remains to be shown

TABLE III. *Mycoplasma* Infection in Heteroploid Cell Cultures Before and After Hypotonic-Antibiotic Treatment.

	Before treatment		1st Antibiotic hypotonic treatment		2nd Antibiotic hypotonic treatment		Condition 12 months after treatment	
Cell line	A	M	A	M	A	M	A	M
Conjunctiva (Chang)	+	+	—	—			—	—
FL amnion (Fogh)	—	+	—	+	—	—	—	—
L-929 (Sanford)	—	+	—	—			—	—*
HeLa-S3-1 (Saltzmann)	—	+	—	—			—	—
HeLa (Gey)	—	+	—	—			—	—
AV-3 (Robbins)	—	+	—	—			—	—
WISH (Hayflick)	—	+	—	—			—	—†
KB (Eagle)	+	+	—	—			—	—
Monkey heart (Salk)	+	+	—	+	—	—	—	—
J-111 (Osgood)	+	+	—	—			—	—
CA-755 (Girardi)	+	+	—	—			—	—
Human heart (Girardi)	—	+	—	+	—	—	—	—

A, aerobic; M, microaerophilic.
* *Mycoplasma* contaminated at 6 mo, recovered *Mycoplasma* free from frozen stock.
† *Mycoplasma* contaminated at 4 mo, recovered *Mycoplasma* free from frozen stock.

TABLE IV. Effect of Hypotonic-Antibiotic Treatment on Sensitivity of Some Heteroploid Mammalian Cell Lines to Vaccinia, Polio 1 and Measles Viruses.

	Vaccinia		Polio 1 Mahoney		Measles	
Cell line	B*	A*	B*	A*	B*	A*
Low *Mycoplasma* contamination†						
FL amnion (Fogh)			8	8.2	4.4	4.2
HeLa (Gey)	5.8	6	7.9	7.8	4	4.3
AV-3 (Robbins)	5.2	5.5	8.1	8.1		
WISH (Hayflick)	6	6.2	7.7	8	4.5	4.5
Human heart (Girardi)	5.9	5.6			4.5	4.3
Gross *Mycoplasma* contamination‡						
KB (Eagle)			8	7.9	3.9	4.6
Conjunctiva (Chang)	5.8	6			4.1	4.7
J-111 (Osgood)	5.4	5.6	7.3	7.9		

* B = Before, A = After antibiotic-hypotonic treatment.
† Less than 100 colonies per million cells. ‡ More than 100 colonies per million cells.
Note: Titers are expressed as $\log_{10}$ of $TCID_{50}$/ml.

whether this selection eliminates cells genetically sensitive to the treatment or if the lost cells simply represent aging cells or cells already damaged by *Mycoplasma* infection.

If this second hypothesis is true, it could well explain why, after this treatment, the growth characteristics, the virus sensitivity, and the morphology of the surviving cells do not appear to deviate from described prototypes.

The results indicate that, when *Mycoplasma* isolation is performed in microaerophylic conditions, simple antibiotic treatment of infected cultures is not an effective cure. This cure appears to be effective only after hypotonic-antibiotic treatment.

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Circumoval Precipitins: Localization by Gel Filtration in Serum of Humans Infected with *S. mansoni.* (29736)

EFRAÍN TORO-GOYCO (Introduced by J. Oliver-González)

Radioisotope Service, Veterans Administration Hospital, San Juan, P. R., and Department of Biochemistry & Nutrition, University of Puerto Rico School of Medicine, San Juan

The circumoval precipitin test(1) is based on the immunochemical reaction occurring between an antigen present in *Schistosoma mansoni* ova and a specific antibody present in the serum of the infected animal or human. The precise location of the antigen in the egg has not been clarified.

The antibody present in humans has been found to migrate in paper electrophoresis with the gamma globulin fraction. A positive circumoval reaction is given by the gamma globulin fraction separated upon fractionation of sera of infected patients by continuous flow paper electrophoresis and starch electrophoresis techniques(2,3). Its concentration in sera seems to be directly related to the severity of the infection(4). No information concerning the size of this antibody, however, was available. Since it was desirable to perform further physical chemical characterization of the antibody, this was attempted using the gel filtration technique which is based on the molecular sieve principle.

Materials and methods. Sephadex G-200, with a particle size of 40-120 μ and a water regain of 20 $\pm$ 2 g/g was obtained from Pharmacia Fine Chemicals, N. Y., and used in preparation of the molecular sieves or columns. These were prepared following the method described by Roskes and Thompson (5). Columns used had dimensions of 2.5 $\times$ 45.0 cm, a void volume of 65 ml, a gel bed of about 160 ml, and rate of flow of 18.0 ml per hour.