Tetrazolium [3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide] Reduction by Mycoplasmas

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We investigated 22 mycoplasma and acholeplasma species for their ability to reduce tetrazolium salts by using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. The test results were evaluated visually, as well as spectrophotometrically, by using an enzyme-linked immunosorbent assay reader. Our results were very similar to the results obtained when the tetrazolium salt reduction assay described by Aluotto et al. was used. However, the MTT reduction assay appeared to be better because it is faster, more objective and sensitive, easier to evaluate, and less expensive; in addition, it allows quantitative determinations. By using regression analysis a linear correlation between formazan production and the number of colony-forming units was demonstrated for all of the species investigated, indicating that the MTT assay can also be used for growth, toxicity, or chemosensitivity tests for the mycoplasma species that are capable of reducing tetrazolium salts.

The ability to reduce tetrazolium salts varies among mycoplasma species and is used as a means of characterization. This ability is usually determined by using the test described by Aluotto et al. (2). In this study we investigated the suitability of the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay for demonstrating tetrazolium reduction by mycoplasmas. The MTT assay is a colorimetric test that was originally developed to determine the survival and growth of eucaryotic cells in proliferation and cytotoxicity tests (8) and was later adapted for monitoring the growth of bone marrow cells in microtiter plate cultures (7).

Mycoplasmas. The mycoplasma species that we used for this study are listed in Table 1. All of the mycoplasmas were cultivated in modified Friis medium (5) under aerobic conditions (5% CO₂) and anaerobic conditions (anaerobic jars; Oxoid) at 37°C. The numbers of colony-forming units were determined by using the method of Albers and Fletscher (1).

MTT assay. Mycoplasmas (100-μl volumes) in the logarithmic growth phase (10⁸ to 10⁹ CFU/ml) were diluted 1:2 with modified Friis medium in 96-well microtiter plates. A 10-μl portion of an MTT solution (5 mg of MTT per ml of phosphate-buffered saline) was added to each well, including two wells that contained only medium (100 μl) (controls). All tests were run in triplicate and preparations were incubated for 4 h at 37°C in an atmosphere containing 5% CO₂. For the spectrophotometric evaluation, 100 μl of a solution containing 20 g of sodium dodecyl sulfate, 50 ml of dimethylformamide, and 50 ml of distilled water (adjusted to pH 4.7) was added to each well, and the plates were incubated for 15 h (overnight) at 37°C to dissolve the formazan crystals. The plates were then read spectrophotometrically with a model SLT 400AT photometer at 540 nm. The results were evaluated by using a modification of a computer program that was written to evaluate enzyme-linked immunosorbent assay data. Briefly, we performed an analysis of variance, a lack of fit test, and a linear regression analysis of the linear part of

the blanked titration curve (log of number of colony-forming units per milliliter versus log of each absorbance value) by using the least-squares method to calculate the slope, intercept, and Pearson correlation coefficient (4). Visual evaluation was possible after 4 h of incubation. Reduction of MTT was manifested by a color change from yellowish to blue that was due to the formation of blue formazan crystals. There was a decrease in the intensity of the blue color that corresponded to the dilution of the mycoplasmas, and the highest dilutions that gave the last visible color change differed for the individual mycoplasmas investigated. The colors of the fluids in the wells after the endpoint, in the control wells (containing medium only), and in the wells which were inoculated with mycoplasmas that were unable to reduce tetrazolium salts remained unchanged. A prolongation of the incubation time to 6, 8, and 10 h neither increased the intensity of the color nor shifted the endpoint of the color change. No increase in staining intensity was observed when we added more than 10 μl of the MTT solution (20 and 30 μl).

The differences in the intensities of the blue color were demonstrated more precisely by spectrophotometric measurements, which were possible after solubilization of the formazan crystals, and subsequent statistical evaluation. The results obtained both by analysis of variance test and the lack of fit test justified the performance of a regression analysis. The coefficients of correlation (range, 0.89 to 0.98) and the slopes of the regression lines (range, -0.93 to -1.04) indicated that the titration plots for all of the mycoplasma species which we tested had a portion with a linear correlation between formazan production and the number of colony-forming units.

The reactions of the 22 mycoplasma and acholeplasma species that we investigated by using the MTT assay and a comparison with the results obtained when we used the test described by Aluotto et al. (2) are shown in Table 1. There was a close correlation between the results of the two methods. However, the MTT assay gave much clearer results, since it permitted quantitative evaluation. The capacities of the mycoplasma species to reduce tetrazolium

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TABLE 1. Tetrazolium reduction by mycoplasma species as determined by the MTT assay and the test of Aluotto et al.

Strain	MTT assay			Test of Aluotto et al.			
	Highest dilution with positive reaction	No. of CFU inoculated		Our study		Previously published data	
		First well	Last positive well	Aerobic conditions	Anaerobic conditions	Aerobic conditions	Anaerobic conditions
<i>Mycoplasma agalactiae</i> PG2	>1:128	1.6×10^{10}	2.5×10^8	— ^a	+	+	+
<i>M. alkalescens</i> DBS80	1:64	2.1×10^7	6.6×10^5	—	+	—	—
<i>M. arginini</i> G230		3.9×10^{10}		—	—	—	+
<i>M. arthritidis</i> PG6		7.5×10^7		—	—	—	—
<i>M. bovigenitalium</i> PG11	>1:128	1.5×10^7	2.3×10^5	—	+	—	+
<i>M. bovirhinis</i> PG43	>1:128	3.0×10^9	4.7×10^7	+	+	+	+
<i>M. bovis</i> Donetta	1:64	8.5×10^6	2.6×10^5	+	+	+	+
<i>M. bovoculi</i> M165/69	1:8	1.0×10^7	2.5×10^6	—	+	+	+
<i>M. capricolum</i> California Kid	1:128	2.8×10^9	4.4×10^7	—	+	+	+
<i>M. edwardii</i> PG24	1:32	6.0×10^7	3.7×10^6	—	+	—	+
<i>M. felis</i> CO	1:16	7.5×10^6	9.4×10^5	—	+	—	+
<i>M. fermentans</i> PG18	1:8	3.8×10^9	9.5×10^8	—	+	—	+
<i>M. gallinarum</i> PG16	1:64	1.2×10^9	3.7×10^7	+	+	+	+
<i>M. gallisepticum</i> PG31	1:128	3.0×10^9	4.7×10^7	+	+	+	+
<i>M. hyorhinis</i> BTS7	1:32	1.5×10^8	9.4×10^6	—	+	+	+
<i>M. hyosynoviae</i> S16		2.5×10^8		—	—	—	—
<i>M. orale</i> CH19299		4.2×10^7		—	—	—	—
<i>M. pneumoniae</i> FH	1:16	2×10^8	2.5×10^7	+	+	+	+
<i>M. pulmonis</i> PG34	1:32	1.2×10^8	7.5×10^6	—	+	—	+
<i>Acholeplasma laidlawii</i> PG8	>1:128	2.7×10^8	4.2×10^6	—	+	ND ^b	ND
<i>A. granularum</i> BTS39	>1:128	1.2×10^9	1.8×10^7	—	+	ND	ND
<i>A. modicum</i> PG49	1:32	3×10^6	1.9×10^5	—	+	ND	ND

^a +, positive reaction; —, negative reaction.^b ND, not determined.

salts were quite different, which was expressed by the minimal number of mycoplasmas that caused clear-cut color changes (Table 1 and Fig. 1). The capacity of individual mycoplasma species to reduce MTT appeared to be a stable property, at least under identical cultivation conditions, and the amount of formazan produced depended on the number

of colony-forming units of the mycoplasmas tested (Fig. 1). Thus, the MTT assay may be used to follow the growth kinetics of mycoplasmas, even species that possess low MTT-reducing capacities. One such species, *Mycoplasma fermentans*, was used to investigate the suitability of the MTT assay as a growth test in more detail.

FIG. 1. MTT-reducing capacities of mycoplasmas. Means $\pm$ standard deviations for three determinations. A regression line has been drawn for each organism. OD 540, optical density at 540 nm. (A) Symbols: ●, *Mycoplasma agalactiae*; ■, *Mycoplasma alkalescens*; ▲, *Mycoplasma bovoculi*; ◆, *Mycoplasma pneumoniae*; ▼, *Mycoplasma edwardii*. (B) Symbols: ●, *Mycoplasma bovigenitalium*; ■, *Mycoplasma bovirhinis*; ▲, *Mycoplasma gallisepticum*; ◆, *Mycoplasma hyorhinis*; ▼, *Acholeplasma laidlawii*. (C) Symbols: ●, *Mycoplasma bovis*; ■, *Mycoplasma capricolum*; ▲, *Mycoplasma felis*; ◆, *Mycoplasma pulmonis*. (D) Symbols: ●, *Mycoplasma gallinarum*; ■, *Mycoplasma fermentans*; ▲, *Acholeplasma modicum*; ◆, *Acholeplasma granularum*.

FIG. 2. MTT reduction during growth of *M. fermentans* D1586. OD 577, optical density at 577 nm. Symbols: □, optical density at 577 nm; ○, protein concentration.

Growth assay with MTT. The MTT growth assay was performed with *M. fermentans* D1586, which was isolated from HL60 cells. *M. fermentans* D1586 was grown in 250-ml tissue culture flasks in GBF-1 medium, which consisted of RPMI 1640 medium (GIBCO) containing 10% fetal calf serum and 10% sonicate of P815 murine mastocytoma cells (or any other murine or human cell line). The cells were adjusted to a concentration of 8×10^6 cells per ml in RPMI 1640 medium and were sonicated until they were completely disrupted (three bursts, 30 s each). The particles were removed by centrifugation at $21,000 \times g$ for 45 min, and the supernatant was passed through a membrane filter (pore size, 0.22 µm; Technomara, Fernwald, Germany) before it was used. Cultures of *M. fermentans* D1586 were incubated at 37°C in an atmosphere containing 5% CO₂. For the MTT assay 6-ml aliquots were withdrawn at various times (Fig. 2) and centrifuged for 45 min at $12,000 \times g$. The resulting mycoplasma pellets were resuspended in 0.6-ml portions of medium, and 100-µl portions of the suspensions were cultured with 10-µl portions of an MTT solution in the wells of a 96-well flat-bottom microtiter plate in a CO₂ incubator for 4 h. Then 120 µl of 5% formic acid in 2-propanol was added to dissolve the blue formazan particles. The contents of each well were thoroughly mixed by using a multichannel micropipette, and the microtiter plate was sonicated for 20 s while it was floating in a sonic bath. For protein determinations 10 ml of culture fluid was withdrawn and centrifuged as described above. The mycoplasma sediment was washed twice in physiological saline and finally taken up in distilled water for protein determination as described by Lowry et al.

(6). The results of this investigation are shown in Fig. 2, which shows that the MTT-reducing capacity of *M. fermentans* went through a maximum, whereas cell mass, as determined by protein content, reached a plateau. These results confirm previous observations on *Mycoplasma pneumoniae* (3).

In this investigation we found that the MTT assay is suitable for investigating mycoplasmas for their ability to reduce tetrazolium salts. There was a good correspondence between the results of the MTT assay and the results of the test described by Aluotto et al. (2). However, the MTT assay has some remarkable advantages. It is much faster (several hours, compared with several days), more objective (allowing quantitative determinations), and cheaper with regard to material costs than the test described by Aluotto et al. (2). Since most mycoplasma species reduce tetrazolium salts (Table 1), this characteristic has less value for the taxonomy and identification of mycoplasmas than previously thought (9). However, the MTT assay can be used to follow the growth kinetics of mycoplasmas. This indicates that the MTT assay can be used as a growth, toxicity, or chemosensitivity assay for mycoplasmas that are able to reduce tetrazolium salts, as well as a tetrazolium reduction assay.

REFERENCES

1. Albers, A. C., and R. D. Flötscher. 1982. Simple method for quantitation of viable mycoplasmas. *Appl. Environ. Microbiol.* 43:958-960.
2. Aluotto, B. B., R. G. Wittler, C. O. Williams, and J. E. Faber. 1970. Standardized bacteriologic techniques for the characterization of mycoplasma species. *Int. J. Syst. Bacteriol.* 20:35-58.
3. Bredt, W. 1976. Estimation of *Mycoplasma pneumoniae* inoculum size by rate of tetrazolium reduction. *J. Clin. Microbiol.* 4:92-94.
4. Franz, B., B. Warlich, and A. Oltrogge. 1989. ELISA for detection of porcine antibodies against *Erysipelothrix rhusiopathiae* bacteria. *Immunobiology (Suppl.)* 4:134.
5. Kirchhoff, H., and R. Rosengarten. 1984. Isolation of a motile mycoplasma from fish. *J. Gen. Microbiol.* 130:2439-2445.
6. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. L. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193:265-275.
7. Monner, D. A. 1988. An assay for growth of mouse bone marrow cells in microtiter liquid culture using the tetrazolium salt MTT and its application to studies of myelopoiesis. *Immunol. Lett.* 19:261-268.
8. Mosmann, T. 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods* 65:55-63.
9. Subcommittee on the Taxonomy of *Mycoplasmatales*. 1979. Proposal of minimal standards for descriptions of new species of the class *Mollicutes*. *Int. J. Syst. Bacteriol.* 29:172-180.