Interference of \textit{Mycoplasma} Infection in a Gene Expression Study: It Was the Environment and Not the Gene

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We show that short-term exposure to doxycycline, as used in tetracycline-inducible gene expression models, protects cells from stress-induced death in cultures infected with \textit{Mycoplasma arginini}. Coinciding with the expected maximum level of gene activity, antimicrobial effects of tetracyclines might be mistaken for antiapoptotic properties of the expressed gene product.

Tetracycline (Tet)-inducible expression of exogenous genes is of particular interest in the case of genes encoding products that interfere with cell growth (2, 6). The murine Schlafen (Slfn) protein family has repeatedly been reported to have growth-inhibitory properties (3, 11). We introduced the human Slfn5 gene into an interleukin-3 (IL-3)-dependent murine hematopoietic precursor cell line. Ba/F3 cells expressing the reverse Tet transactivator (Ton.BaF.1) (4) were retrovirally infected with a doxycycline-sensitive \textit{Mycoplasma} \textit{arginini} isolate and confirmed by immunoblotting. Expression of Slfn5 was induced by doxycycline (1 μg/ml) and confirmed by immunoblotting. Expression of Slfn5 did not interfere with cell growth in the presence of IL-3. Deprivation of IL-3, however, was significantly better tolerated by the Slfn5-expressing cells than by uninduced controls, suggesting that Slfn5 might exert protective properties under stress conditions.

We next exposed the Ton.Slfn5 clone to X-irradiation and were able to reproduce the survival benefit for the cells in the doxycycline arm. To exclude the (as we believed) theoretical possibility that doxycycline by itself, and not the induced gene, was responsible for the observed cytoprotective effects, we repeated the X-irradiation experiments using the untransduced mother cell line Ton.BaF.1. To our surprise, doxycycline protected these “empty” cells in the same way. Apart from singular reports on neuroprotective effects (1), doxycycline has not been recognized as a substance with direct cytoprotective properties. We therefore hypothesized that antimicrobial activity had protected our cells from stress-induced death and further concluded that cultures might have been contaminated with a doxycycline-sensitive \textit{Mycoplasma} species (5). In fact, mycoplasma infection could be verified in both the Ton.Slfn5 clone and the mother cell line (MycobAlert mycoplasma detection kit; Lonza, Rockland, ME).

Culture isolates were grown for 5 days of incubation at 37°C under anaerobic conditions on both Columbia agar supplemented with 5% sheep blood (Becton Dickinson, Heidelberg, Germany) and A7 mycoplasma agar (bio-Merieux, Marcy l’Etoile, France). Species determination was performed by complete 16S rRNA gene and 16S-23S intergenic spacer (IS) sequence analyses as well as a growth inhibition test (8). Sequence analyses of the isolate’s 16S rRNA gene and 16S-23S IS revealed the highest sequence similarity values to \textit{Mycoplasma arginini} G230T (GenBank accession no. AF125581 and AY737013) (99% and 98%, respectively). A growth inhibition test using antisera raised against the closest relatives (\textit{M. arginini}, \textit{M. gateae}, \textit{M. alkalescens}, and \textit{M. canadense}) finally identified the isolate as \textit{M. arginini}.

Susceptibility to doxycycline was determined by MIC testing using a broth macrodilution assay in which growth was denoted by a color change of the medium (arginine hydrolysis, alkaline shift from pH 6.8 to pH 7.6) in the presence of the phenol red pH indicator. Frozen aliquots (~80°C) of stock cultures of the \textit{M. arginini} isolate and \textit{M. arginini} G230T were thawed and serially diluted to determine CFU on SP-4 agar plates (12). On the day of the assay, stock cultures were thawed and diluted in SP-4 broth (pH 6.8) containing arginine (0.1%, wt/vol) to yield 5 × 10^6 CFU/ml, which was confirmed by CFU determination on agar plates. Inoculated broth was dispensed in 1-ml aliquots into glass tubes, and then 1 ml of serially 2-fold-diluted doxycycline was added to each tube to obtain 12 concentrations ranging from 0.031 to 64 μg/ml. Further, a sterility control (uninoculated broth, pH 6.8), an endpoint control (uninoculated broth, pH 7.6), and a growth control (doxycycline-free inoculated broth) were included. Tubes were incubated at 37°C under atmospheric conditions, and final MICs were recorded 5 days later as the lowest doxycycline concentration showing no change in color of the medium. Assays were performed in duplicate.

In all broth macrodilution assays, growth of the \textit{M. arginini} isolate and \textit{M. arginini} G230T was inhibited by doxycycline at a...
MIC of 0.5 μg/ml. It could thus be concluded that the doxycycline dose used in our Tet-inducible system (1 μg/ml) exerted potent antimicrobial activity against the culture contaminant. Cultures were treated with MycoKill antibiotics (PAA, Pasching, Austria) according to the manufacturer’s recommendations. Successful eradication was confirmed by broad-range real-time PCR targeting the 16S rRNA gene, culture, and a MycoAlert test (9).

To further document how mycoplasma infection might interfere with Tet-inducible gene expression studies, we aimed at reproducing our incidental observations in a prospective experimental setting. Contaminated and mycoplasma-free Ba/F3 cells were grown in RPMI 1640 medium with 10% fetal calf serum (FCS) and IL-3 (1 ng/ml) at 37°C and 5% CO₂. Slfn5 expression was induced by the addition of doxycycline (1 μg/ml). For starvation experiments, cultures were deprived of IL-3.
for 21 h. X-irradiation experiments were performed using an IBL 437 C X-ray machine (CIS bio international, Paris, France). The number of viable cells was determined daily. Total cell counts were measured on a Sysmex XE-2100 hematology analyzer (Sysmex Corporation, Kobe, Japan). Viability was estimated by propidium iodide staining on a FACSCalibur instrument (Becton Dickinson, Franklin Lakes, NJ). All experiments were performed in triplicate. Results were considered to be significantly different when the \( P \) value was less than 0.05 (two-tailed Student’s \( t \) test). Under normal culture conditions, there was no significant difference in cell growth—neither between mycoplasma-free and -contaminated cultures nor between cells with and without expression of Slfn5. However, after exposure to either starvation or X-irradiation, cells induced to express Slfn5 exhibited a significant survival advantage in contaminated cultures. They constantly regrew after both kinds of stress, whereas uninduced controls did not recover during the observation period. In contrast, mycoplasma-free cultures always recovered, independently of whether or not they expressed Slfn5. Detailed results are shown in Fig. 1.

Mycoplasma infections can produce a myriad of effects on contaminated cell lines (5). The elimination of mycoplasma from cell cultures by antibiotic treatment usually takes a few weeks. We show that short-term exposure to doxycycline, as used in Tet-inducible gene expression models, may exert cytotoxic protective activities in cultures with a sensitive contaminant. Coinciding with the expected maximum level of gene activity, antimicrobial effects might be mistaken for antiapoptotic properties of the expressed gene product. There has been some concern that doxycycline might falsify the effects of an induced protein by acting cytotoxic with certain cell lines (13), but this has not yet been discussed as a protective bias. The problem of mycoplasma contamination has been recognized for decades. It is, of course, general knowledge that regular testing of cell cultures is indispensable for good scientific practice, especially to detect insidious species that do not cause overt cytopathology. Nevertheless, even recent studies report infection rates of cell lines in the range of 15 to 35% (5, 7, 10). Our observations add a new perspective to the jigsaw puzzle of possible scientific errors caused by contamination of cell lines with mycoplasma and once more stress the importance of regular mycoplasma screening in tissue culture.

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REFERENCES
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Volume 76, no. 23, p. 7867–7869, 2010. Page 7868, legend to Fig. 1, line 2: “(day 1)” should read “(day −1).”
Page 7869, column 1, line 31: “this” should read “it.”