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Detection of Mycoplasma Infection of Mammalian Cells

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ABSTRACT

Mycoplasma infection was detected in cultures of COS cells with a novel, simple assay that detects the conversion of arginine to citrulline by the enzyme, arginine deiminase, specific to all species of mycoplasma. Transfection of COS cells was inhibited in mycoplasma-infected cells, a phenomenon that was readily reversed by treatment with a mycoplasma removal agent. Cultures of cells used for transfection should be regularly monitored for evidence of mycoplasma by assay of arginine deiminase activity or by other methods.

INTRODUCTION

Mycoplasma are parasitic prokaryotic organisms that reside in endosomes of mammalian cells. They are incapable of peptidoglycan synthesis and thereby lack a rigid cell wall, which makes them resistant to penicillin-type antibiotics that are often added to cell culture medium (8). The effects of mycoplasma infection of mammalian cells varies depending on the species of mycoplasma, the degree of infection and the cell type. More specific effects of infection include preferential incorporation of nucleic acid precursors into mycoplasmal cells at the expense of host cells (5), secretion of collagenase by the host cell (7), interference with signal transduction (3), altered levels of IgE and transferrin receptors in basophilic leukemia cells (2) and increased secretion of soluble interleukin-2 receptor (6). Most, if not all, mycoplasma utilize arginine as an energy source mediated by the enzyme (Enzyme Commission No. EC3.5.3.6) arginine deiminase (9,10). A side effect of the metabolism of arginine by this organism is the production of ammonia along with citrulline. It has been suggested that arginine deiminase activity maintains endosomes at a pH that blocks cycling through the lysosomal pathway. Thus, a major deleterious effect of mycoplasma may be interference with normal vesicular trafficking in cells. Most transfection methods involve endocytosis of plasmid DNA complexed with various agents, such as DEAE-dextran, $CaPO_4$, lipids, and so on. This is a particularly important issue with respect to transient transfections.

MATERIALS AND METHODS

Growth and transfection of COS cells was performed exactly as described (4). COS cells were stained with the 4′,6-diamidino-2-phenylindole (DAPI) according to the manufacturers instructions (Sigma Chemical, St. Louis, MO, USA). Briefly, cells were washed with methanol followed by the addition of 10 mg/mL DAPI in methanol, incubated for 15 min at room temperature, washed twice with methanol and visualized with a fluorescent microscope using a 40× oil immersion objective. Mycoplasma removal agent was obtained from ICN Biomedicals (Costa Mesa, CA, USA) and was used as directed.

RESULTS AND DISCUSSION

We have observed that, in some cultures of COS cells, transfection efficiency was drastically decreased, and the cells grew slowly. However, it was otherwise not obvious that the cells were not healthy. Apart from the slow growth, the cells plated efficiently and maintained a flattened fibroblastic morphology. Since we suspected mycoplasma infection, we investigated this possibility by assaying COS cells for arginine deiminase activity by means of a novel assay method. The assay is very similar to a routine assay used in this laboratory for nitric oxide synthase and depends upon the conversion of radioactive arginine (which is charged) to citrulline (which is uncharged) (1). The simplicity of the citrulline assay allows hundreds of enzyme assays to be done in a few hours. A 10-cm dish of COS cells were prepared by brief sonication (10 s) in 2 mL phosphate-buffered saline (PBS) with a probe sonicator. For routine assays, 25 µL of cell extract

(25–100 μg protein) were incubated with 100 000 cpm [3H]arginine (50 Ci/mmol; Amersham, Arlington Heights, IL, USA) in 12- × 75-mm borosilicate disposable culture tubes for 30 min at 22°C. The reaction was terminated by addition of 2 mL of water to the reaction test tube; this mixture was applied to 0.5-mL columns of Dowex AG-50WX-8 (Na+ form; Sigma Chemical). The resin was prepared in bulk by stirring it for 1 h in water, to which was added NaOH pellets until the pH exceeded 12.0. It was then neutralized by extensive water rinses and then stored as a suspension until needed. Radioactive citrulline in the flow-through was collected and quantitated by scintillation counting. In cells that transfected with poor efficiency, cell extracts contained an arginine deiminase activi-

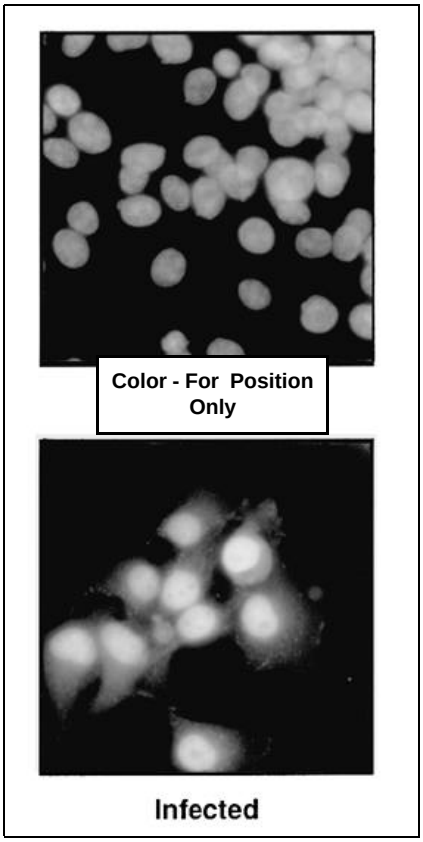

Figure 1. DAPI staining of uninfected and mycoplasma-infected COS cells. COS cells were plated on glass cover slips and incubated for 24 h. They were then fixed rapidly in methanol and stained with DAPI at a dilution of 1:1000 in methanol. After mounting the coverslips on slides, the cells were visualized on a fluorescence microscope at 40× with an oil immersion lens and then photographed.

ty of approximately 54 nmol per 10^6 cells per h. In a control culture, approximately 2 nmol per 10^6 cells per h were detected. The enzyme activity was blocked by arginine analogs, such as nitroarginine and methylarginine (data not shown). The identity of the flow-through counts as citrulline was confirmed by separation of a sample on a reverse-phase HPLC column (data not shown). We estimate that activity can be detected in as few as 5000 cells by using more cell extract, more radioactivity and extended incubation times. However, cells used for transfection are usually not in short supply, so we normally use a 10-cm dish of cells, i.e., about 3×10^6 cells. Furthermore, by the time mycoplasma infection is even suspected, the amount of arginine deiminase activity in cell lysates is very large. Hence, the number of counts obtained from infected cells is routinely in the vicinity of 50–60 000 cpm. In this sense, our assay gives an immediate indication of infection that is unmistakable in commonly used cells such as COS, CHO, HEK293, etc. It should be pointed out that this assay would be compromised in cells with a functioning urea cycle. This is not, however, an issue with cells normally used for transfection purposes.

The presence of mycoplasma in the cells was confirmed further by staining cells with DAPI, a stain that fluoresces blue when bound to DNA. Only the nucleus of uninfected cells was stained by this dye (Figure 1). In contrast, considerable cytoplasmic staining was seen in the cytoplasm of cells that transfected poorly, a pattern indicative of mycoplasma infection. To establish whether mycoplasma infection was in fact the cause of low transfection efficiency, we treated the infected culture for 7 days with mycoplasma removal agent at 50 µg/mL. Then both infected and treated cells were transfected with cDNAs encoding nicotinic receptor subunits, α4 and β2, by means of an adenovirus-mediated transfection method (4). In control cells, specific binding of ^{3}H-acetylcholine binding averaged 312 fmol per 10-cm dish of cells. In infected cells, the level of binding was reduced to 74 fmol (Figure 2). We conclude from these experiments that mycoplasma has a profoundly deleterious effect on transfection of COS cells. These data emphasize the need to exercise continued vigilance concerning this problem, since infected cells show little obvious evidence of infection. The routine use of simple mycoplasma detection assays, such as the arginine deiminase assay, or DAPI staining, is strongly recommended. Other assays that are based on the use of polymerase chain reaction (PCR) primers require access to a thermal cycler and use expensive enzymes and reagents. In contrast, the present assay requires very basic skills and equipment present in most laboratories.

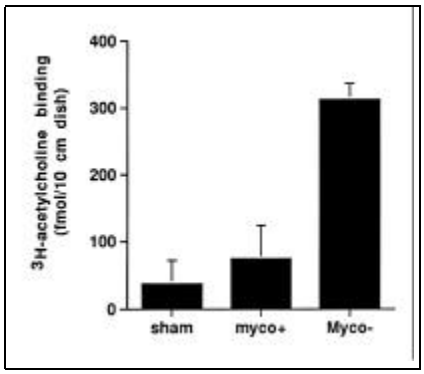

Figure 2. Effect of mycoplasma infection on the expression of α4β2 neuronal nicotinic acetylcholine receptor in COS cells. Data are expressed as fmol of ^{3}H-acetylcholine bound specifically and are the means of three independent experiments ± standard error of the mean (SEM). Specific acetylcholine binding was defined as the difference in bound radioactivity between aliquots incubated with ^{3}H-acetylcholine alone or supplemented with 1 mM nicotine. Myco+ represents mycoplasma-contaminated cells, myco- represents mycoplasma-free cells.

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