

# Antimycoplasma Properties and Application in Cell Culture of of the biological reagent Mynox<sup>®</sup>

DIRK VOLLENBROICH, GEORG PAULI,<sup>2</sup> MUHSIN ÖZEL,<sup>2</sup> AND JOACHIM VATER<sup>3</sup>

*Minerva Biolabs GmbH, Köpenicker Straße 325, 12555 Berlin,<sup>1</sup>*

*Robert Koch-Institut, Fachbereich Virologie, 13353 Berlin,<sup>2</sup> and*

*Max-Volmer-Institut für Biophysikalische Chemie und Biochemie, Fachgebiet Biochemie und Molekulare  
Biologie, Technische Universität Berlin, 10587 Berlin,<sup>3</sup> Germany*

**Mycoplasma contamination of cell cultures is a serious and widespread problem. Contaminated cultures are frequently discarded or the contamination ignored since treatment with standard antibiotics is undesirable, time-consuming, and an ineffective means to eliminate mycoplasmas. Minerva Biolabs developed an innovative method based on the biological reagent Mynox<sup>®</sup>. A single treatment over one passage led to complete removal of viable *Mycoplasma hyorhinitis* cells from various adherent cell lines, and *Mycoplasma orale* was removed from nonadherent human T-lymphoid cell lines by a single treatment. This effect was monitored by a DNA fluorescence test, an enzyme-linked immunosorbent assay, and two different PCR methods. Disintegration of the mycoplasma membranes as observed by electron microscopy indicated the mode of action of Mynox<sup>®</sup>. Disintegration is obviously due to a physicochemical interaction of the membrane-active Mynox<sup>®</sup> with the outer part of the lipid membrane bilayer, which causes permeability changes and at higher concentrations leads finally to disintegration of the mycoplasma membrane system. The low cytotoxicity of Mynox<sup>®</sup> for mammalian cells permits specific inactivation of mycoplasmas without significant deleterious effects on cell metabolism and the proliferation rate in cell culture. A fast and simple method for complete and permanent inactivation of mycoplasmas in mammalian monolayer and suspension cell cultures is described.**

Mycoplasmas are causative agents of serious diseases of humans and animals, such as acute respiratory inflammation (including pneumonia) and diseases of the urogenital tract, and seem to be cofactors in the pathogenesis of AIDS (1, 15). These smallest free-living organisms are parasites of eukaryotic cells and are one of the major contaminants that affect tissue culture cells. The most prevalent agents that do this are the mollicute species *Mycoplasma orale* (a human species), *Mycoplasma hyorhinitis* (a porcine species), *Mycoplasma arginini* (a bovine species), and *Acholeplasma laidlawii* (a bovine species) (7). Contaminating mycoplasmas affect a variety of cellular processes and cell morphology, deplete the nutrients in the growth medium, and interfere with virus replication (2, 13). For both biological and ecological reasons, it is important to eliminate these agents from cell cultures used for basic research, diagnosis, and biotechnological production. The most effective procedure for eliminating, inactivating, or suppressing mycoplasmas in cell cultures is treatment with antibiotics (3, 12, 14). In general, antibiotic therapies do not result in longlasting successful decontamination, and undesirable side effects on eukaryotic cells due to cytotoxic effects and the development of resistant mycoplasma strains have been observed (12, 14). Mycoplasmas lack a cell wall but are encircled by a three-layer cytoplasmic membrane, so that antibiotics such as the penicillins, which are common additives in cell culture media and interfere with murein formation in cell walls, are not effective against them.

A new commercially available antimycoplasma agent with novel modes of action is urgently required. In this article we show the mode of Mynox<sup>®</sup> action and the efficiency of the method for eliminating mycoplasmas from adherent and nonadherent mammalian cells.

## MATERIALS AND METHODS

**Cells and culture conditions.** All cell lines investigated for mycoplasma elimination were continuous cell lines cultured in medium without any antibiotic at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub> in air. Adherent cell lines ML (mink lung), 293 (human embryonal kidney), CV1 (African green monkey kidney), and Hep2 (human larynx carcinoma) were cultivated in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Uxbridge, Great Britain) supplemented with 5% heat-inactivated fetal calf serum (FCS) (ICN Pharmaceuticals Inc., Irvine, Calif.) in petri dishes (Nunc, Roskilde, Denmark). Suspension cell lines MT-4 (human T-cell leukemia virus type 1 transformed), Molt 4 clone 8 (Molt 4/8), and H9, all of which are human T-lymphoid cell lines, were grown in RPMI 1640 medium (ICN) supplemented with 10% (vol/vol) FCS and 2 mM L-glutamine in tissue culture flasks (Nunc). All suspension cell lines and adherent cell line CV1 were subcultured once a week, and all other adherent cell lines were subcultured twice a week.

**Cytotoxicity assay.** The cytotoxicity of Mynox<sup>®</sup> (Minerva Biolabs, Berlin, Germany) for the adherent cell lines were determined by the crystal violet dye uptake assay by using the method described by Flick and Gifford (7). Adherent cells (approximately 10<sup>5</sup> cells/ml) were grown on microtiter plates (200 µl of cell suspension/well) with serial dilutions of Mynox<sup>®</sup> mixed with DMEM supplemented with 5% (vol/vol) FCS. After the control culture was confluent, the cells were fixed with 1% glutaraldehyde, stained with crystal violet, washed with H<sub>2</sub>O, and dried. The dye was dissolved in 100 µl of ethanolwater-acetic acid (50:49.9:0.1). A<sub>550</sub> values were determined with a microplate reader. The proliferation rates of the nonadherent cell lines after treatment with Mynox<sup>®</sup> were determined by the colorimetric tetrazolium dye reduction assay and the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (8). The yellow compound MTT (Sigma) is

reduced by mitochondrial dehydrogenases to the water-insoluble blue compound formazan, depending on the viability of the cells. A 20- $\mu$ l portion of a solution of MTT (5 mg/ml in PBS) was added to every well. The plate was incubated for 4 h at 37°C in a CO<sub>2</sub> incubator. After incubation 150  $\mu$ l of medium was removed from every well without disturbing the cell clusters. A 100- $\mu$ l portion of acidified isopropanol (2 ml of concentrated HCl added to 500 ml of isopropanol) was added to each sample, and the preparations were mixed thoroughly on a plate shaker with the cells containing formazan crystals. After all of the crystals were dissolved, the A<sub>550</sub> values were determined with a microplate reader.

#### **Mycoplasma detection.**

**(i) Cytochemical staining of DNA with DAPI.** The cell cultures used for cytochemical staining of DNA with 49,6-diamidino-2-phenylindole (DAPI) (Boehringer, Mannheim, Germany) (11) were grown on coverslips in petri dishes to 70% confluence. The culture medium was removed, and the cells were fixed with methanol at room temperature. The cells were incubated for 5 min at 37°C with a DAPI staining solution (1  $\mu$ g of DAPI per ml of methanol) and rinsed with methanol. Using PBS as the mounting medium, we examined the cells with a fluorescence microscope fitted with a Zeiss filter combination consisting of type BP 365 and 520-560 filters. Mycoplasmas appeared as bright yellow-green spots against a dark cytoplasmic background next to the fluorescence signal of the stained nuclear DNA of the mammalian cells.

**(ii) Immunological detection: enzyme-linked immunosorbent assay (ELISA).** Immunological detection and identification of *M. orale*, *M. hyorhinis*, *A. Laidlawii*, and *M. arginini* were performed with a commercially available mycoplasma detection kit (Boehringer). Biotinylated polyclonal antibodies directed against specific mycoplasma antigens were reacted with the cells. Binding of the antibodies was visualized by the streptavidin-alkaline phosphatase assay. After enzymatic hydrolysis of 4-nitrophenyl phosphate, the yellow nitrophenol product was quantified with a microplate reader (model EAR 400 AT; SLT-Labinstruments, Grödig, Austria) at a wavelength of 405 nm.

**(iii) PCR amplification of mycoplasma rRNA.** The templates used for mycoplasma PCR were extracts of cell-free culture media prepared by boiling. A 100- $\mu$ l portion of a cell culture supernatant was transferred into a sterile Eppendorf tube and boiled for 5 min. The tube was centrifuged briefly. A mycoplasma PCR primer set obtained from Stratagene (La Jolla, Calif.) was used to differentiate mycoplasma types, and the VenorGeM<sup>®</sup> Mycoplasma Detection Kit (Minerva Biolabs GmbH, Germany) was used for fast non-species specific detection of mycoplasmas. Each final PCR mixture (total volume, 50  $\mu$ l) contained 2  $\mu$ l of template and 48  $\mu$ l of amplification mixture containing 1.25 U of *Thermus aquaticus* DNA polymerase (InViTek, Berlin, Germany). Each reaction mixture was overlaid with 2 drops of mineral oil. PCR amplification was performed with a Perkin-Elmer thermal cycler. The initial 5-min denaturation step at 94°C was followed by a 1.75-min annealing step at 55°C. Next there was a 3-min primer extension step at 72°C, followed by a 45-s denaturation step at 94°C and a 1.75-min annealing step at 55°C. The remaining 40 cycles each consisted of extension for 3 min at 72°C, denaturation for 45 s at 94°C, and annealing for 45 s at 55°C. The posttreatment steps consisted of 10 min at 72°C and then 10 min at 27°C. The amplified PCR products were separated by standard agarose gel electrophoresis in 2% (wt/vol) agarose gels and were visualized by ethidium bromide staining.

**Electron microscopy.** ML cells were propagated in DMEM supplemented with 5% FCS. After the culture was confluent, Mynox<sup>®</sup> was added to the medium in various volumes around the volume given in the protocol. After 1 h of incubation at 37°C, the cells were fixed with 2.5% glutaraldehyde in PBS and concentrated by gentle centrifugation after successive washes with PBS. The fixed pellets were enclosed in an agar block, dehydrated in ethanol, and embedded in Epon by standard techniques (5). Ultrathin sections were examined at 80 kV with a Zeiss model EM 902 electron microscope.

**Mycoplasma elimination procedures.** Reproducible decontamination of adherent and nonadherent cell lines was performed as follows. The Mynox<sup>®</sup> used in the procedures described below was directly added to the medium.

**(i) Adherent cell lines.** 1 \* 10<sup>4</sup> to 1 \* 10<sup>6</sup> of freshly trypsinized cells were transferred into a sterile cell culture flask or petri dish containing standard cell culture medium with 200  $\mu$ l Mynox<sup>®</sup>. The total volume of the culture was equal to 5 ml. The final FCS concentration was 5% v/v. It was important to ensure that Mynox<sup>®</sup> was already present in the culture medium before the cells were added. After 2 hours of incubation under normal growth conditions, the elimination mix was removed by discarding the supernatant, the cells were then overlaid with standard cell culture medium.

For the most effective method, the cells were maintained in the elimination mixture for one entire passage (approximately 3 to 8 days) under normal growth conditions. The medium containing Mynox<sup>®</sup> was then removed and the cells were subcultured in standard medium as normal.

**(ii) Suspension cell lines.** Depending on the proliferation rate, 1 \* 10<sup>4</sup> to 1 \* 10<sup>5</sup> cells of a suspension cell line were transferred into a centrifuge tube containing the elimination mixture (1.6 ml of standard cell culture medium with 10 % v/v FCS, 1.6 ml trypsin:EDTA (0.125%:5mM) in phosphate buffered saline (PBS), and 200  $\mu$ l Mynox<sup>®</sup>). The mixture was vortexed and shaken gently for 30 min at room temperature. After centrifugation at 600 \* g for 5 min, the supernatant was discarded. The cells were resuspended in Mynox<sup>®</sup>-free standard cell culture medium.

For a more effective method, a subcultivation in the presence of Mynox<sup>®</sup> for 1 passage was possible. The cells were resuspended in 5 ml of cell culture medium containing 5% v/v FCS and 150  $\mu$ l of Mynox<sup>®</sup>. The cells were incubated in this medium for 3 days in a culture flask under normal growth conditions and the cells were then separated from the elimination mixture by centrifugation. The cells were then subcultured in Mynox<sup>®</sup>-free growth medium.

## RESULTS

**Effect of Mynox® on cell proliferation.** We investigated the antimycoplasma properties of Mynox® on ML cells and observed a significant increase in cell proliferation, and the confluent culture was vital and healthy and had less contrast than an untreated culture. When mycoplasma-free cell cultures were treated with Mynox®, no changes in morphology or proliferation rate could be detected. In order to investigate the effect of Mynox® in more detail, several cell lines were collected from different sources and tested for mycoplasma infection. Positive cell lines were exposed to Mynox® at various concentrations. The cytotoxic effect of Mynox® was measured by the crystal violet technique for adherent cell lines or by the MTT assay for suspension cell lines (Fig. 1). Cell lines CV1, Hep2, and ML showed low cytotoxic effects of 4, 10, and 32 %, respectively, at the recommended Mynox® concentration. Only cell line 293 showed almost 65 % cytotoxicity. At concentrations greater than two times the suggested working concentration no cells survived after one passage. The loss of cell material was compensated rapidly due to a massive improvement in the proliferation rates of the cells after complete removal of the contaminants.

**Mycoplasma detection and differentiation.** Stock cultures and Mynox®-treated cultures were tested for mycoplasma infection by the DAPI staining method, which showed that all of the stock cultures were mycoplasma positive (Fig. 2a), while the cultures treated with the antibiotic were free of mycoplasmas (Fig. 2b). The fluorescence DAPI test is only useful for screening, as only massive mycoplasma contaminations can be detected. In order to enhance the detection limit for mycoplasmas, the cultures were tested by the highly sensitive PCR and ELISA techniques two passages after treatment at the earliest. The species of contaminating mycoplasmas were identified by the ELISA for all of the cell lines investigated. The mycoplasma species residing in adherent cell lines ML, 293, Hep2, and CV1 was identified as identified as *M. hyorhinis*. The mycoplasmas in suspension cell lines Molt 4/8, H9, and MT-4 belonged to *M. orale*. The Stratagene PCR method permits differentiation between PCR products derived from different mycoplasma species. Figure 3 shows typical fingerprint results obtained with two strains of mycoplasmas. For *M. orale* in MT-4, Molt 4/8, and H9 cells, one band at 650 bp was observed, and for *M. hyorhinis* in ML, 293, Hep2, and CV1 cells, four bands at 700, 600, 250, and 150 bp were detected. No double infections were observed with either method.

**Biological activity of Mynox® against mycoplasmas.** To understand the mode of action of Mynox®, we treated a confluent ML cell culture that was highly contaminated with *M. hyorhinis* with Mynox® at several concentrations and investigated the effects by transmission electron microscopy (Fig. 4). Mycoplasmas incubated in a cell culture without Mynox® were visible as intact particles at the surfaces of the ML cells. After incubation of mycoplasmas with half the suggested working concentrations at 37°C for 1 h, we observed formation of small holes in the mycoplasma membrane and swelling of the particles. Especially mycoplasmas attached to the cell surface were directly affected by Mynox®. At an optimal Mynox® concentration bursting of the particles was induced. Also, mycoplasmas which were hidden in pockets and clefts of the cell membrane began to disintegrate. Finally, at a Mynox® concentration double the suggested working concentration disruption of the mycoplasma lipid bilayer included total disintegration of the membrane systems, which led to bursting of all microorganisms. The efficiency of the treatment is shown in Table 1 and Fig. 3.

**Mycoplasma elimination procedure.** On the basis of the observations described above, we developed and tested a procedure for removing mycoplasmas from mammalian suspension and monolayer cells by using the lytic effect of Mynox® on these organisms. After Mynox® treatment, cells had been cultivated in the absence of antibiotics so that a low level of infection would be detectable after two passages by each of the methods. Contamination with mycoplasmas was assessed by using at least three different procedures. For all cell lines treatment with Mynox® for two passages did completely eliminate mycoplasma contamination. The viability of the cells 293 was significantly lower than the viability of CV1 and Hep2 cells after one passage with Mynox®. Higher Mynox® concentration led to complete removal of mycoplasmas, but the cells were either dead or seriously damaged, which led to unacceptably low proliferation rates. Therefore, we recommend using the suggested protocol as a standard elimination procedure for adherent cell lines in order to be sure that all cell lines are devoid of mycoplasma infections.

Using the results obtained with the adherent cell lines, we determined the duration of Mynox® exposure and the concentrations required for treatment of suspension cell lines Molt 4/8, H9, and MT-4, as described above. By double treatment with Mynox® we obtained complete elimination of mycoplasmas in all cell lines. Double the amount of Mynox® led either to cell death or to the reduction of proliferation rates below acceptable levels after one to two passages.

To eliminate the possibility that mycoplasmas hidden in intercellular spaces, as well as in pockets and clefts of the cell membrane, would escape contact with the drug, we used trypsin to detach the cells from each other and to smooth the cell surfaces. Under these conditions our results were nicely reproducible.

We tested the reemergence of residual mycoplasmas by cultivating the cells for up to 28 passages. As Table 1 shows in the periods investigated all of the cell lines could be cultivated so that they were free of contamination. In no case was any permanent growth inhibition of mammalian cells detected. The surviving cells regained the normal rate of growth and compensated for the loss in cell numbers after one to two passages. The initial cell density and the FCS concentration were important factors for the success of this procedure. Higher cell densities and FCS concentrations in the reaction mixture decreased the efficiency of elimination of mycoplasmas, but lower concentrations decreased the level of viability.

## DISCUSSION

Our investigation of the mycoplasmacidal effect of Mynox<sup>®</sup> was initiated by the observation that mycoplasma-contaminated adherent and nonadherent cells exhibited improved proliferation rates and changes in morphology. These effects of Mynox<sup>®</sup> are obviously caused by a disruption of the plasma membrane, which is its primary site of activity. Mynox<sup>®</sup> causes leakage, at higher concentrations it leads to complete disintegration of the membrane systems, and finally it causes the mycoplasmas to burst. Eradication of the contaminants resulted in native morphology and a native proliferation rate of the mammalian cells.

Additional studies with artificial membranes, protoplasts, and eukaryotic cells (data not shown) demonstrated that Mynox<sup>®</sup> binds readily to cell membranes with a high degree of selectivity, depending on the membrane lipid composition, anchoring in the lipid bilayer, showing high affinities to cholesterol and phospholipids. In *Mycoplasma* species cholesterol was found at levels comparable to those in the plasma membranes of eukaryotic cells (25 to 30% [wt/wt] of the total membrane lipids) (9, 10). The higher levels of membrane phospholipids (especially phosphatidylglycerol, phosphatidylcholine, and phosphatidylethanolamine) found in *Mycoplasma* cells compared with eukaryotic cells may result in the greater susceptibility of mycoplasma membranes to Mynox<sup>®</sup>. Mynox<sup>®</sup> is active against *M. hyorhina* and *M. orale* and more than 10 other mycoplasma species (data not shown). Mynox<sup>®</sup> interacts with the mycoplasma membrane, inducing an osmotic influx of medium and ultimately complete disruption of the cells. Our observations of the lytic effect of Mynox<sup>®</sup> on mycoplasmas were utilized to develop an efficient procedure for elimination of mycoplasmas from mammalian cell cultures. All adherent cell lines and suspension cells tested could be successfully cleansed of two of the most common mycoplasmas associated with such cell cultures, *M. hyorhina* and *M. orale*, respectively. The efficiency of the mycoplasma elimination procedure which we developed was demonstrated by several highly sensitive techniques. No reemergence of the contaminants was detected, which meant that the mycoplasmas were completely eradicated and not merely arrested in growth by a bacteriostatic effect of the drug. All cultures from which *Mycoplasma* species were eliminated were grown under conditions under which recontamination by other contaminated cell cultures was not possible (i.e., in a separate incubator or laminar airflow biohazard cabinet).

Compared with other mycoplasma elimination protocols performed with antibiotics, such as ciprofloxacin and enrofloxacin (quinolone derivatives; trade names, Ciprobay and Baytril, respectively), tiamulin and minocycline (pleuromutilin and tetracycline derivative, respectively; combined to form the commercially available product BM-Cyclin), or Mycoplasma Removal Agent (a 4-oxo-quinoline-3-carboxyl acid derivative) (3, 6, 12, 14), the method described here has the advantage of being more effective. Therefore, cells do not have to be protected against reemergence of contaminants, and time-consuming and labor-intensive replenishment of the antibiotic during cultivation is not necessary. Indeed, the only antibiotics able to eliminate mycoplasmas in a cell culture without producing high cytotoxicity are the tetracyclines and the fluoroquinolones, both of which are known to penetrate cells and inhibit mycoplasma metabolism (1, 3, 14). The mycoplasmas blocked in growth by these antibiotics are removed from cell cultures mainly by dilution. In some cases removal is incomplete and mycoplasmas appear again after a few passages. In contrast to these mycoplasmastatic drugs, Mynox<sup>®</sup> does not inhibit growth of the mycoplasmas; instead, due to its physicochemical mechanism of action, it kills the contaminating mycoplasmas directly. Therefore, only a short treatment is necessary for complete disintegration of these organisms. On the basis of the mode of action of Mynox<sup>®</sup> it is expected that mycoplasmas will not develop resistance to this drug, which is a major advantage compared with the antibiotics mentioned above.

The damaging effects of the agent on plasma membranes were mitigated in a culture medium with a high serum content, probably as a result of the high competitive binding capacity of the large amounts of proteins or lipids in the medium. Therefore, Mynox<sup>®</sup> is not useful for eliminating mycoplasmas from samples with high protein contents. However, the novel mycoplasma inactivation procedure developed certainly is of great medical and biotechnological interest. Mycoplasmas are capable of altering many properties of mammalian cells and parameters measured in cell cultures, which leads to unreliable results. Mynox<sup>®</sup> can be used to keep growth, morphology, and metabolism in a natural state. In addition, Mynox<sup>®</sup> is well-suited to eliminate mycoplasma infections in biotechnological and pharmaceutical products, including vaccines, therapeutics, care products, and diagnostics derived from in vitro systems, as advised by most governmental regulatory agencies.

## TABLES AND FIGURES

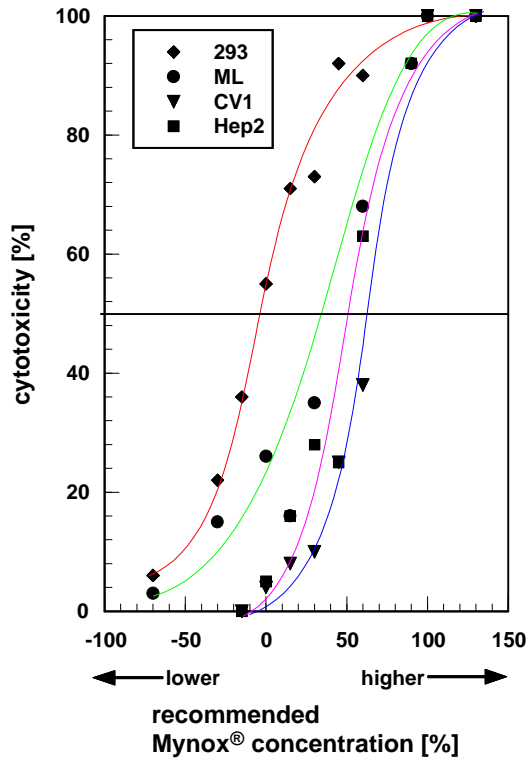


FIG. 1. Cytotoxic effects of Mynox<sup>®</sup> on different cell lines. The inhibitory effects of different doses of Mynox<sup>®</sup> on cell growth were determined by the crystal violet assay. Cell lines ML, 293, Hep2, and CV1 were treated with Mynox<sup>®</sup> at various concentrations. No Mynox<sup>®</sup> was added to the control culture. The percent growth reduction was calculated from the extinction difference between a Mynox<sup>®</sup>-treated cell culture and the control.

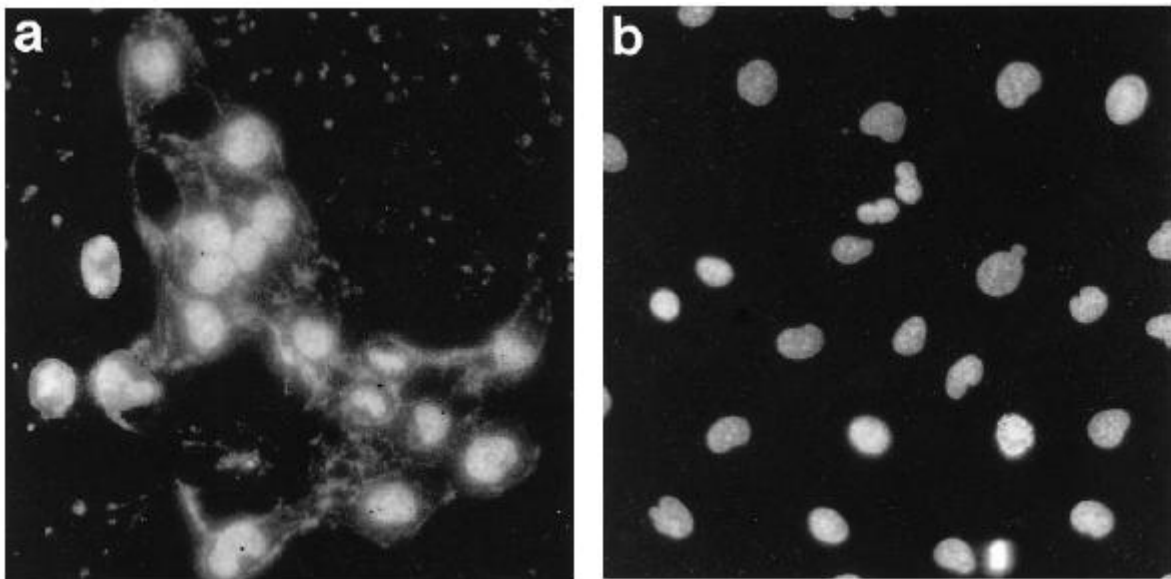


FIG. 2. DNA fluorescence staining of Mynox<sup>®</sup>-treated cells with DAPI: ML cells heavily contaminated with *M. hyorhinis* (a) and mycoplasma-free cultures (b) after treatment with Mynox<sup>®</sup>. Mycoplasmas were detected after the DNA in the culture was stained with the fluorochrome dye DAPI; they appear as small fluorescent spots against a dark background in the cytoplasm and intercellular spaces.

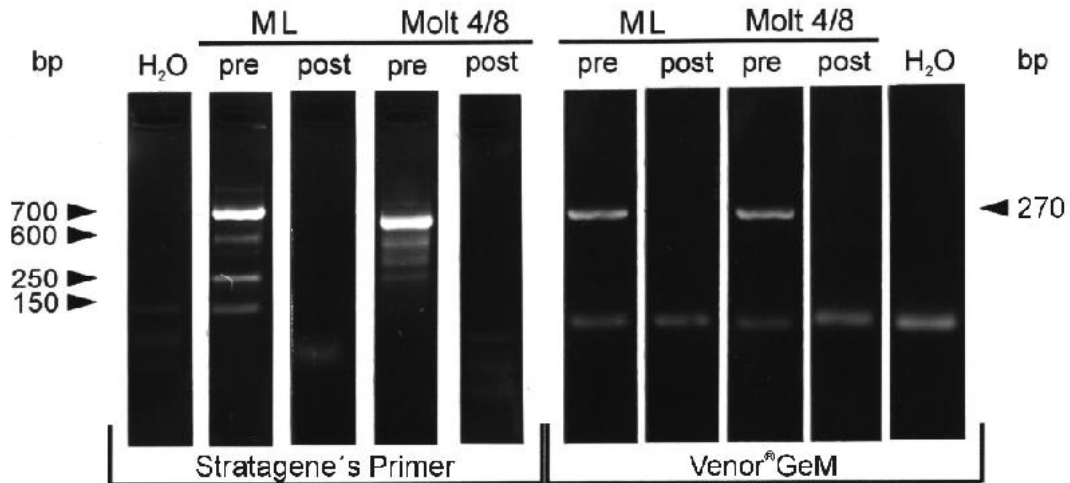


FIG. 3. Gel electrophoretic analysis of PCR amplification products of cell culture supernatants before and after Mynox<sup>®</sup> treatment. Only mycoplasma rRNA was amplified by PCR when the type-specific Stratagene mycoplasma primer set was used (a) or the species-specific VenorGeM<sup>®</sup> was used (b). Templates obtained from the culture medium containing Mynox<sup>®</sup>-treated cells (post) produced no PCR signals. The Stratagene primer set revealed the *M. hyorhinis*-specific pattern (700, 600, 250, and 150 bp) for the untreated ML culture (pre) and an *M. orale*-specific pattern (650 bp) for the untreated Molt 4/8 culture (pre). The VenorGeM<sup>®</sup> was considered mycoplasma positive if a single 270-bp product was amplified.

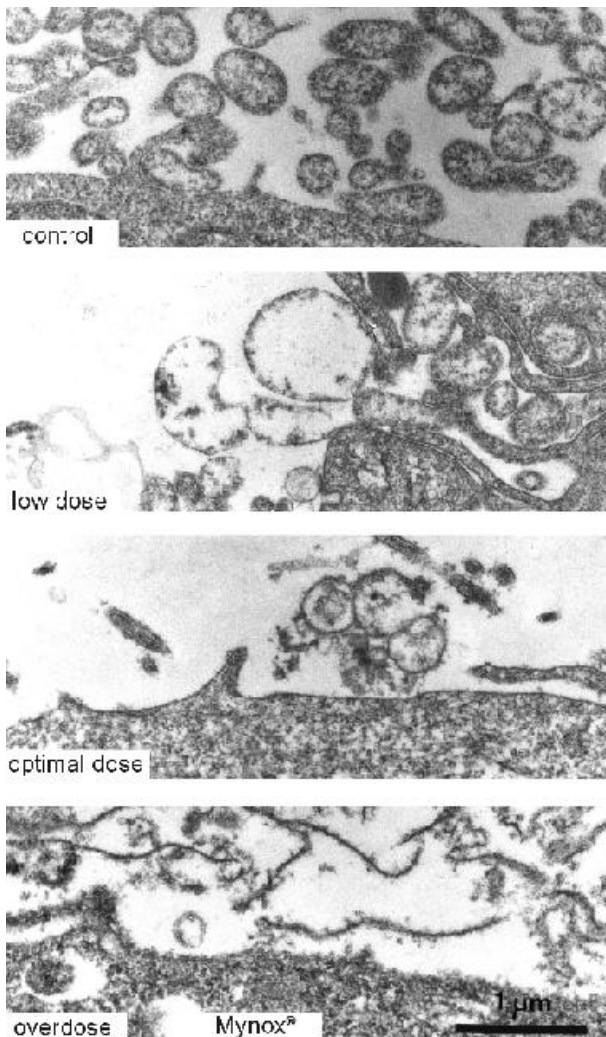


FIG. 4. Thin-section electron micrographs of mycoplasma-contaminated ML cells before and after addition of Mynox<sup>®</sup>. When an ML cell culture that was highly contaminated with *M. hyorhinis* was confluent, Mynox<sup>®</sup> was added and the preparations were incubated for 60 min at 37°C. No Mynox<sup>®</sup> was added to the control culture. Interaction of the membrane-active Mynox<sup>®</sup> with the outer part of the lipid membrane bilayer induced permeability changes. At the higher concentrations the drug finally caused the mycoplasma membrane system to burst.

TABLE 1. Mycoplasma elimination by Mynox<sup>®a</sup>

Cell line	Detection assay	Passage(s) tested for mycoplasma contamination
Adherent cell lines		
ML	DAPI	2, 5, 15, 20
	ELISA	6
	PCR (Stratagene method) <sup>b</sup>	2, 5, 15, 20
	PCR (van Kuppeveld method) <sup>c</sup>	2, 5, 15, 20
CV1	DAPI	2, 3, 4
	ELISA	3
	PCR (Stratagene method)	3, 5
	PCR (van Kuppeveld method)	2, 5
293	DAPI	8, 17, 28
	PCR (Stratagene method)	4, 12, 28
	PCR (van Kuppeveld method)	4, 12, 14, 18, 25, 28
Hep2	DAPI	8, 20
	PCR (Stratagene method)	2, 5
	PCR (van Kuppeveld method)	4, 9, 16, 20
Suspension cell lines		
Molt 4/8	ELISA	10
	PCR (Stratagene method)	4, 9, 15, 20
	PCR (van Kuppeveld method)	3, 4, 6, 9, 15, 20
MT-4	ELISA	10
	PCR (Stratagene method)	4, 6, 20
	PCR (van Kuppeveld method)	3, 4, 6, 10, 16, 20
H9	ELISA	10
	PCR (Stratagene method)	3, 5, 9, 20
	PCR (van Kuppeveld method)	3, 5, 7, 9, 13, 16, 20

*a* The effectiveness of the elimination process was controlled during several cell passages by using different mycoplasma detection methods. All of the cultures tested after Mynox<sup>®</sup> treatment were free of contaminants. In the cell passages tested no mycoplasma recontamination occurred during cultivation. *b* PCR performed with primers obtained from Stratagene. *c* PCR performed with the VenorGeM<sup>®</sup> Mycoplasma Detection Kit

## ACKNOWLEDGMENTS

We thank E. Nissen for helpful discussions and K. Dunkelmann for technical assistance in the preparation of the electron micrographs.

## REFERENCES

- Blanchard, A., and L. Montagnier. 1994. AIDS-associated mycoplasmas. *Annu. Rev. Microbiol.* **48**:687–712.
- Chowdhury, M. I. H., T. Munakata, Y. Koyanagi, S. Arai, and N. Yamamoto. 1994. Mycoplasma stimulates HIV-1 expression from acutely- and dormant-infected promonocyte/monoblastoid cell lines. *Arch. Virol.* **139**:431–438.
- Fleckenstein, E., C. C. Uphoff, and H. G. Drexler. 1994. Effective treatment of mycoplasma contamination in cell lines with enrofloxacin (Baytril). *Leukemia* **8**:1424–1434.
- Flick, D. A., and G. E. Gifford. 1984. Comparison of in vitro cell cytotoxic assays for tumor necrosis factor. *J. Immunol. Methods* **68**:167–175.
- Gelderblom, H. R., E. H. S. Hausmann, M. O'zel, G. Pauli, and M. A. Koch. 1987. Fine structure of human immunodeficiency virus (HIV) and immunolocalization of structural proteins. *Virology* **156**:171–176.
- Hay, R. J., M. L. Macy, and T. R. Chen. 1989. Mycoplasma infections of cultured cells. *Nature* **339**:487–488.
- McGarrity, G. J., V. Vanaman, and J. Sarama. 1978. Methods of prevention, control, and elimination of mycoplasma infection, p. 213–241. *In* G. J. McGarrity, D. G. Murphy, and W. W. Nichols (ed.), *Mycoplasma infection of cell cultures*. Plenum Press, New York, N.Y.
- Mosmann, T. 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assay. *J. Immunol. Methods* **65**:55–63.
- Razin, S. 1978. The mycoplasmas. *Microbiol. Rev.* **42**:414–470.
- Rottem, S. 1980. Membrane lipids of mycoplasmas. *Biochim. Biophys. Acta* **604**:65–90.
- Russell, W. C., C. Newman, and D. H. Williamson. 1975. A simple cytochemical technique for demonstration of DNA in cells infected with mycoplasma and viruses. *Nature* **253**:461–462.
- Schmidt, J., and V. Erffle. 1984. Elimination of mycoplasmas from cell cultures and establishment of mycoplasma-free cell lines. *Exp. Cell Res.* **152**:565–570.
- Stanbridge, E. J., and C.-J. Doersen. 1978. Some effects that mycoplasmas have upon their infected host, p. 119–134. *In* G. J. McGarrity, D. G. Murphy, and W. W. Nichols (ed.), *Mycoplasma infection of cell cultures*. Plenum Press, New York, N.Y.
- Uphoff, C. C., S. M. Gignac, and H. G. Drexler. 1992. Mycoplasma contamination in human leukemia cell lines. II. Elimination with various antibiotics. *J. Immunol. Methods* **149**:55–62.
- World Health Organization. 1993. Report of the WHO Meeting on the Development of Vaginal Microbicides for the Prevention of Heterosexual Transmission of HIV. World Health Organization, Geneva, Switzerland.