Role of Mycoplasma Infection in the Cytopathic Effect Induced by Human Immunodeficiency Virus Type 1 in Infected Cell Lines

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AIDS is characterized primarily by a profound alteration in the function and number of T4 lymphocytes, resulting directly or indirectly from infection with human immunodeficiency virus type 1 (HIV-1) or HIV-2 but were unable to block virus replication. A contaminating mycoplasma was isolated from our CEM c13 cells and identified as a strain of Mycoplasma fermentans. Following infection of lymphoblastoid (CEM) or promonocytic (U937 and THP1) cell lines with HIV-1, cytopathic effect was observed only in association with mycoplasmal contamination. Moreover, HIV-1 infection of U937 cells after experimental inoculation with a human isolate of M. fermentans led to pronounced cell killing. We have verified that this effect is not merely an artifact caused by arginine and/or glucose depletion in the cell culture medium. These results confirm that mollicutes, in particular M. fermentans, are able to act synergistically with HIV-1 to kill infected cells in some in vitro systems.

MATERIALS AND METHODS

Cells and viruses. Cell lines CEM (c111 or c113) and H9 are clones of a human T-lymphoblastoid CCRF-CEM cell line (ATCC CCL119) and T-lymphoma HuT78 cell line (ATCC TIB162), respectively, selected for their ability to efficiently replicate HIV. U937 (ATCC CRL1593) is a human promonocytic cell line, and THP1 (ATCC TIB202) is a human monocytic leukemia cell line. Cells were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 2 mM glutamine, 100 IU of penicillin per ml, and 100 μg of streptomycin per ml. The antibiotics tested were mycoplasma removal agent, pefloxacin (Peflacin), minocycline (Myncin), doxycycline (Vibramycin), clindamycin (Dalacin), erythromycin (Erythrocin), and chloramphenicol (Tifomycin or Salnicol). Mycoplasma removal agent is a quinolone of unknown structure supplied by Flow Laboratories (McLean, Va.). The other antibiotics were purchased from Sigma (St. Louis, Mo.) or obtained from the central pharmacy of the Rhône-Poulenc Rorer Research Center (CRVA, Vitry-Alfortville, France). Culture assays were performed as previously described (12, 36) in microtitration plates (96 wells) or in 24-well plates, as indicated.

Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll gradient centrifugation. In 50-ml tubes, 33 ml of blood was layered on top of 17 ml of Ficoll-containing lymphocyte separation medium (MSL; Eurobio, Paris, France). The tubes were centrifuged for 30 min at 400 × g at 4°C. Then, PBMC were collected and washed in complete culture medium. After trypan blue dye exclusion evaluation of the number of living cells, the PBMC were used immediately or placed in cryotubes (5 × 10^6 cells per tube) and frozen in liquid nitrogen. They were cultivated in supplemented RPMI 1640 medium containing interleukin-2 (10 U/ml; Lymphocult, Biogen). The LAVBr strain of HIV-1 was used throughout this study. Virus stocks were obtained from Diagnostic Pasteur.
HIV-seronegative donor then the blood of a
in days centrifugation was assayed then enzyme counted in nation tetraacetic acid, poly(rA)-oligo(dT) (0.5
was infectious supernatant necessary, HIV
mM + 10^6 cells/(A540
of cell culture
1640 RPMI medium supplemented in
with 50 ml of the antibiotics (dissolved in 1%
dimethylformamide–99% PBS) or PBS alone. The cultures
were then infected by the addition of 10^6 ml of HIV-1,
containing 50 to 250 times the minimal dose necessary
to infect a CEM cell culture. Mock-infected cells received 100
ml of supplemented RPMI medium. Cells were incubated at
37°C in a 5% CO2 incubator. At day 7 after HIV infection
and then every 3 or 4 days, supernatant (60 ml) was removed for
the RT assay (as described below) and 40 µl of the cell
supersediment was transferred to another microplate containing
210 µl of fresh medium supplemented with the antibiotic to be
tested. The remaining cells in 100 ml were used for the cell
viability assay with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphe-
nylethyltetrazolium bromide (MTT) assay (29, 36). Viability was
calculated by using the formula: % viable cells = [(A_{540} of
HIV-infected + treated cells)/(A_{540} of mock-infected +
treated cells)] x 100.

The cytoidal effect of mycoplasmas on HIV-infected cells
was studied in 24-well microtiter plates. In this case, 2 ml of
cell suspension (8 x 10^4 cells per ml) was infected with
HIV-1 (same dose as above) in 500 µl of supplemented RPMI
1640. Mock-infected cells received only supplemented RPMI. An aliquot of cell culture supernatant was removed
every 3 or 4 days to measure RT activity, p24 antigen
concentration, and cell viability. The remaining cells were
diluted to continue the incubation.

In vitro RT assay. As a marker of HIV replication, RT
activity in cell supernatants was determined as described
previously (36). Briefly, 50 µl of supernatant was incubated for
1 h at 37°C with 50 µl of RT reaction cocktail, containing
50 mM Tris-HCl (pH 7.8), 50 mM KCl, 5 mM MgCl2, 5 mM
dithiothreitol, 0.05% Triton X-100, 0.5 mM EGTA (ethylene
glycol tetraacetic acid), poly(rA)-oligo(dT) (0.5 A_{540}/ml), and
[U^3H]TTP (0.11 MBq [1 µM]). The polynucleotides were then
precipitated with 20 µl of 60% trichloroacetic acid in 120 mM
Na2P2O7, and the samples were filtered on glass fiber filters
with a Skatron cell harvester (Skatron Instruments Inc.,
Sterling, Va.). The filters were dried, and radioactivity was
counted in a beta scintillation counter (LKB, Uppsala,
Sweden).

Virus replication was measured in parallel by the determi-
nation of p24 antigen levels in culture supernatants with an
enzyme immunoassay kit (Du Pont, Wilmington, Del.).

Mycoplasma isolation, detection, and characterization. A
clinical mycoplasma isolate was obtained from experiments
that were originally designed to recover clinical HIV isolates
from the blood of HIV-seropositive individuals. PBMC (6 x
10^6 cells) from HIV-seropositive patients were prepared by
Ficoll centrifugation as described above and cultivated for 3
days in 6 ml of supplemented RPMI 1640. These cells were
then cocultivated with 3 x 10^6 PBMC from a healthy
HIV-seronegative donor that were previously activated for 3
days with 0.1% phytohemagglutinin (Difco Laboratories,
Detroit, Mich.) in supplemented interleukin-2-containing
RPMI 1640. Every 3 or 4 days, cells were evaluated for
viability (trypan blue dye exclusion), virus production was measured (RT assay and p24 antigen detection), and the
culture medium was renewed. After 11 days of coculture,
supernatants from these samples were mixed with SF-4
mycoplasma medium (38) (1:9, vol/vol), incubated at 37°C,
and checked daily for mycoplasma growth.

The M. fermentans strain AOU that was isolated during
this study and the M. pirum strain BER (25) are available
from the Collection of the Institut Pasteur (Paris, France).
Mycoplasma contamination or experimental infection of cell
lines, PBMC, HIV-1 stocks, and fetal calf serum was rou-
tinely tested by four different methods: culture in SF-4
mycoplasma medium (38), fluorescence staining with 4',6-
diamine-2'-phenylindole (DAPI) after coculture (3 and 6
days) with 3T6 indicator cells (32), detection of adenosine
phosphorylase in cell culture supernatants (5), and microbi-
ological cultures (1). In order to avoid cross-contamination
of cell cultures, manipulations were performed essentially
by the method of McGarity et al. (21). Moreover, contam-
inated and noncontaminated biological materials were man-
ipulated in separate laminar flow hoods and incubated in
dedicated incubators.

Identification and characterization of mycoplasmas were
performed by the serological and molecular approaches
described by others (35). M. fermentans-specific antisera
was kindly provided by J. G. Tully (National Institute of
Allergy and Infectious Diseases, Frederick, Md.).

Preparation of mycoplasma extracts. Mycoplasma extracts
were prepared from three strains (M. fermentans AOU, M.
pirum BER, and M. hominis type strain PG21). Mycoplasma
cells were recovered from broth culture by centrifugation
(12,000 x g, 15 min, 4°C) and washed twice in PBS. Cell lysis
was achieved by sonication (four times for 30 s each at 0°C)
of a mycoplasma suspension containing 5 x 10^4 color-
changing units (CCU) per ml in PBS. The crude cytoplasmic
fraction was separated from the membrane fraction by
centrifugation (180,000 x g, 1 h, 4°C), and the cytoplasmic
fraction was collected. The effect of mycoplasma extracts on
RT activity was measured by incubation of these extracts (1
or 5 µl) with concentrated virus (1 or 5 µl) and RT reaction
cocktail (50 µl). The final volume was adjusted to 100 µl with
PBS, and the mixture was incubated for 1 h at 37°C. The
enzymatic reaction was then stopped by the addition of 20 µl
of 60% trichloroacetic acid in 120 mM Na2P2O7. The precipi-
tate was filtered and counted as described above.

PCR analysis of mycoplasma strains. Mycoplasma lysates
were prepared as described previously (4), and DNA ampli-
fication was performed by the method of Saiki et al. (34). The
oligonucleotides that were used as primers for the polymer-
ase chain reaction (PCR) were 5'-GAAATTCTTTAT
TGAGTGGCTC-3' and 5'-AACCCCTTTCGAAAATGC
CGG-3'. These primers were chosen according to a nucleo-
tide sequence that was reported to be specific for M.
fermentans (M. incognitus) (17). DNA was amplified for 35
cycles at 95°C for 20 s, 58°C for 1 min, and 72°C for 1 min.
The products of the reaction were resolved in a composite
gel of 2% NuSieve and 1% Seakem agaroses (FMC Corp.,
Rockland, Maine) in Tris-borate buffer and stained in ethid-
ium bromide solution.

RESULTS

Antibiotic effects on HIV-associated CPE. To confirm and
extend our previous results with tetracycline analogs (12),
we investigated the effect of several other antibiotics on
CEM ci13 viability after HIV-1 infection. Syncytia were observed in cultures between 4 and 10 days after HIV-1 infection. After this time, cytotoxic effects were not seen with effective doses of antibiotics in the cultures, although infected cells continued to produce HIV-1 (Fig. 1). In contrast, there was substantial cell death in HIV-infected cultures supplemented with too little or inactive antibiotic.

The most active compound for providing cell viability protection was mycoplasma removal agent, a quinolone of unknown structure (Fig. 1A). This compound reduced by 50% the CPE associated with HIV-1 replication at a dose of 0.001 μg/ml (50% effective concentration [EC_{50}]). Pefloxacin, another quinolone antibiotic, displayed an EC_{50} of 0.03 μg/ml (Fig. 1B). For minocycline (Fig. 1C) and doxycycline (not shown), the two most active tetracycline analogs, the EC_{50} was 0.5 to 1 μg/ml. Clindamycin (Fig. 1D) was active only above 3 μg/ml. Erythromycin (Fig. 1E) and chloramphenicol (Fig. 1F) were not able to provide complete protection at nontoxic doses. All compounds were toxic for noninfected cells at doses higher than those reported in Fig. 1.

Throughout these experiments, AZT (3′-azido-2′,3′-dideoxythymidine), added at a final concentration of 1 μM, inhibited both cell killing and viral replication, as expected for this antiviral agent (not shown). By contrast, none of the tested antibiotics active in our assays has antiviral activity (i.e., inhibition of RT activity or p24 antigen production).

**Mycoplasma detection and identification.** As the antibiotics in the previous experiments are active against mycoplasmas, we examined the CEM cell line for mycoplasma contamination. Using DAPI staining with the 3T6 indicator cell line (32), measurement of adenosine phosphorlyase activity (5), and microbiological techniques (1), we confirmed that our particular clone of CEM cells was mycoplasma contaminated. In addition, the mollicute was isolated in SP-4 medium. This mollicute was able to hydrolyze arginine and ferment glucose, and its growth was inhibited with *M. fermentans*-specific antiserum. When analyzed by PCR with specific oligonucleotide primers for *M. fermentans* (*M. incognitus*), the DNA from this strain produced a specific amplified product (168 bp, lower band; Fig. 2, lane 2). As described previously for the strain of *M. fermentans* called *incognitus* (17), another slower-migrating band (about 185 bp) was also obtained. Although no explanation has yet been provided for the amplification of two DNA fragments, one can suggest that this is due to the existence of multiple copies (that may be heterogeneous) of this DNA sequence within the genome of *M. fermentans* (17). The CEM cell line was therefore contaminated with *M. fermentans*, which is a common contaminant of cell cultures. Indeed, about 5% of the mollicutes that contaminate cell cultures were reported to belong to this species (22).

A mycoplasma was similarly isolated from U937 cells. This mollicute hydrolyzed arginine but did not ferment glucose, indicating that it is not a member of the species *M. fermentans*. In addition, PCR amplification of DNA from this mycoplasma with the *M. fermentans*-specific primers described above was not successful (Fig. 2). However, only some strains of *M. fermentans* (K7, AOU, and the one isolated from CEM) could be amplified with these primers. The type strain PG18 of the same species was weakly positive. Controls with primers specific for *M. pneumoniae* (3) and *Ureaplasma urealyticum* (4) were also negative (not shown).

**Mycoplasma reduction of RT activity.** The enhancement of RT activity by treatment of HIV-1-infected cells with anti-biotics could be explained by the reduction of mycoplasma products interfering with the in vitro RT assay, as reported in two recent articles (18, 39). To examine this possibility, a concentrated HIV-1 suspension was incubated with crude mycoplasma extracts and nucleic acids.

The effect of culture medium on the incorporation of tritiated TTP in the RT assay was examined. HIV-1 (25 μl) was mixed with 25 μl of PBS, RPMI 1640, or SP-4 medium and assayed for RT. The relative radioactivity obtained in the three reactions was 100, 62, and 14%, respectively. Therefore, subsequent mycoplasma extracts were prepared in PBS.

Extracts from only some mycoplasmas inhibited the RT assay. As shown in Table 1, the extract from *M. hominis* did not inhibit the RT reaction. On the other hand, extracts from *M. fermentans* and *M. pirum* inhibited the RT reaction. The addition of 5 μl of extracts from these two species inhibited 99% of the RT activity. Preincubation of the *M. fermentans* extract for 1 h at 60°C partly abolished this inhibition (only 40% inhibition compared with 99%, as reported in Table 1).

**Mycoplasma contamination and HIV-induced CPE in various cell lines.** To determine whether HIV-induced CPE in non-T-lymphocytic cell lines can also be dependent on the presence of mycoplasmas, we chose monocytic (THP1 and U937) cell lines that were contaminated or not with mycoplasmas. The selected cell lines were infected with HIV-1 cell supernatant assessed to be mycoplasma-free. The results, summarized in Table 2, confirmed that, in these in vitro systems, CPE and extensive culture destruction (more than 50% cell lysis) were observed only when HIV-1 infection was associated with mycoplasma contamination. When mycoplasma-free cells were infected with HIV-1, only a slight and transient decrease in viability (less than 20%) was measured.

However, we also observed that infection of H9 cells with HIV-1 (strain LAV_{Br}) did not produce CPE and that experimental contamination with *M. fermentans* did not change this feature. In a similar preliminary experiment with normal PBMC, no enhancement of HIV-associated CPE was obtained in the presence of added *M. fermentans* (data not shown).

**Isolation of *M. fermentans* from cultured PBMC from an HIV-seropositive patient.** A mycoplasma was isolated from an 11-day-old culture of PBMC from an HIV-seropositive and asymptomatic individual. SP-4 medium was inoculated with the supernatant of this culture, and within 6 days, mycoplasma growth was indicated (color shift from red to yellow). However, no mycoplasma was recovered from six similar cultures of PBMC from other HIV-seropositive patients that were processed at the same time. The strain isolated (AOU) hydrolyzed arginine and fermented glucose and was identified as a strain of *M. fermentans* by growth inhibition with *M. fermentans*-specific antiserum. In addition, specific amplified products were obtained by PCR analysis with *M. fermentans*-specific primers (Fig. 2, lane 4).

**Influence of mycoplasma infection on CPE induced by HIV-1.** We tested the ability of *M. fermentans* AOU to act synergistically with HIV-1 to produce cell lysis. For that purpose, mycoplasma-free U937 cells were inoculated with 5 × 10^5 CCU of strain AOU. Addition of the mycoplasma induced a cytostatic effect on days 4 and 7 postinoculation with no gross cytotoxicity (fewer cells than in the control mycoplasma-free U937 but without killed cells, as measured by trypan blue dye exclusion). After 14 days, mycoplasmas could still be recovered from the experimentally infected cell cultures by using SP-4 medium, while uninfected cells re-
FIG. 1. Activity of antibiotics on CPE and RT activity in CEM c113 infected with HIV-1. The results were obtained on day 18 after HIV-1 infection. Each value represents the average of three independent points. The antibiotics tested were mycoplasma removal agent (MRA) (A), pefloxacin (B), minocycline (C), clindamycin (D), erythromycin (E), and chloramphenicol (F). The concentration of the antibiotics is given on the x axis. CPE is measured as a percentage of viable cells (left y axis, open circles), and RT activity in the culture supernatant is shown on the right y axis (solid circles).
mained negative through 28 days of culture. RT activity and p24 antigen were not detected in any of these cultures, confirming that strain AOU was clearly free of HIV. Then, mycoplasma-free cells and mycoplasma-infected cells were inoculated with mycoplasma-free HIV-1 (100% the minimum dose needed to infect CEM cl13 cells) or medium alone. The results shown in Table 3 demonstrate that lysis of U937 occurred only when mycoplasmas were present.

*M. fermentans* can hydrolyze arginine and ferment glucose, and a trivial explanation for lysis of U937 cells would be that mycoplasma growth depleted arginine and/or glucose from the medium (23), resulting in a more pronounced HIV-induced CPE. Therefore, in a control experiment, arginine and glucose were periodically added to the culture medium after HIV-1 infection of both *M. fermentans*-contaminated CEM cells and mycoplasma-contaminated U937 cells. Addition of these nutrients did not alter the CPE (not shown), indicating that cell death cannot be attributed to depletion of arginine and/or glucose.

**DISCUSSION**

Our results indicate that some species of mycoplasmas, in particular *M. fermentans*, can act synergistically with HIV-1 to promote extensive cell death in some in vitro cell systems, such as CEM cells and U937 cells. However, enhancement of CPE associated with the presence of mycoplasmas in HIV-infected cells cannot be extended as a general rule, because it was not found with H9 cells and normal PBMC.

Antibiotics from the fluoroquinolone and, to a lesser degree, from the tetracycline families were able to inhibit the in vitro cytoidal effect of HIV-1 without affecting viral replication. Although antibiotics greatly reduced single-cell lysis, they did not inhibit lysis of cells involved in syncytium formation. This observation differs from data published by Lo et al., who reported a reduction in syncytium formation when their mycoplasma strain was added to HIV-1-infected lymphocytic A3.01 cells (18). This discrepancy could be due to the fact that the strains of virus and mycoplasma and the cell line differ from the ones that were used in our study. However, these authors (18) have also observed an increased CPE associated with the presence of *M. fermentans* in HIV-infected cells, which is consistent with our results.

Two different species of mycoplasmas, including *M. fermentans*, were isolated from our cell cultures and found to enhance cell killing associated with HIV-1 infection in lymphocytic (CEM cl13) and in promonocytic (U937 and THP1) cell lines.

We also confirmed that the contamination of HIV-infected cell cultures with some mycoplasma species (*M. fermentans* and *M. pirum*, but not *M. hominis*) interferes with RT detection in cell supernatants. One likely hypothesis is that enzymes, such as nucleases, produced by the mycoplasmas are interfering with the RT assay.

Several mechanisms may explain the enhancement of CPE associated with the presence of mycoplasmas. First, mycoplasma contamination could result, for instance, in alteration of receptor expression as a consequence of mycoplasma adhesion (28, 33). This modification could facilitate the attachment of HIV to and/or the entry of HIV into the cells. Second, mycoplasmas have previously been shown to increase the secretion of certain soluble factors, such as cytokines and, in particular, tumor necrosis factor (13), which is known to activate HIV replication (31) and promote

<table>
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<th>TABLE 1. Influence of mycoplasma extract on RT activity</th>
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<td><em>M. fermentans</em></td>
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* RT activity is expressed as cpm per sample. The value obtained when no mycoplasma extract was added was taken as 100% (control).

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<th>TABLE 3. Influence of <em>M. fermentans</em> AOU on CPE induced by HIV-1 in U937 cells</th>
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* U937 cells were experimentally infected (+) or not (−) with *M. fermentans* prior to HIV-1 infection.

* See Table 2, footnote b.

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<th>TABLE 2. Influence of mycoplasma infection on HIV-1-induced CPE in different cell lines</th>
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* The cell lines were shown to be contaminated with mycoplasmas (+) or not contaminated (−) prior to HIV-1 infection.

b RT activity in the culture supernatant.

c ND, not determined.
mycoplasma, and M. fermentans were also analyzed: strain AOU (lane 4), PG185 (lane 5), and K7 (lane 6). The DNA size markers (lanes 1 and 7) are HaeIII-digested φX174 DNA fragments.

greater CPE in some cell lines (11, 20). Such a mechanism has been suggested in a recent article (6), in which it was reported that a crude extract from the mollicute *Acholeplasma laidlawii* increased HIV-1 replication in chronically infected Molt-4 cells but not in H9 or in U937 cells. However, in our laboratory, the addition of live *M. fermentans* to chronically HIV-1 infected CEM cells did not increase HIV replication, and *M. fermentans* (strain AOU) contamination of different cell lines (CEM and U937) was not concomitant with tumor necrosis factor production (data not shown). Therefore, the tumor necrosis factor hypothesis seems unable to explain by itself the mycoplasmal CPE enhancement. Finally, mycoplasmas could act as cofactors that have been shown to transactivate the HIV-1 long terminal repeat and to increase virus replication in vitro (26). Increased virus production and cytolysis were also associated with mycoplasma contamination of normal mononuclear cells that were infected with cytomegalovirus (37). However, mycoplasma contamination does not necessarily increase virus-induced cytolysis. Pratt et al. (30) reported that poxvirus infection of mycoplasma-infected cells produced a lower virus yield than in non-contaminated cells. But when MRK13 (rabbit kidney) cells were infected with the Lister strain of vaccinia virus, mycoplasma contamination induced increased virus replication.

In conclusion, the observations by Lo et al. (17, 18) and the isolation of mycoplasmas from the blood of patients with AIDS in our laboratory (24, 25; this study) indicate that mycoplasmas may be another opportunistic in immunocompromised patients and perhaps act as a cofactor in accelerating the development of AIDS. This hypothesis cannot be ruled out and deserves further investigation (8). Moreover, some in vitro biological properties of HIVs and of CD4⁺ cells (28, 33) can clearly be modified by mycoplasmas. Because these microorganisms are common cell culture contaminants, the effects of mycoplasmas must be considered carefully in HIV studies.

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