*Free Rad. Res.*, Vol. 23, No. 3, pp. 197–212
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# RELEASE OF HYDROGEN PEROXIDE FROM HUMAN T CELL LINES AND NORMAL LYMPHOCYTES CO-INFECTED WITH HIV-1 AND MYCOPLASMA

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*(Received October 4th, 1994; in revised form, January 4th, 1995)*

Human T-cell lines and normal lymphocytes persistently or acutely co-infected with the human immunodeficiency virus type 1 (HIV-1) and mycoplasmas were found to release hydrogen peroxide ($H_2O_2$), a likely cause of oxidative stress in these cells. The spectrofluorometric measurement of $H_2O_2$ release from these cells, using the scopoletin fluorescence quenching technique, gave values of 16–84 p moles/$10^6$ cells/min. In CEM cells, $H_2O_2$ was released only when acutely co-infected with HIV-1 and mycoplasmas, and not when infected with either organism alone. Anti-mycoplasmal antibiotics strongly reduced $H_2O_2$ release, and improved cell viability without blocking virus replication. These results suggest that the simultaneous infection by HIV-I and mycoplasma leads to the release of $H_2O_2$, a toxic and potentially lethal metabolite, which *in vivo* may contribute to HIV-1 pathogenicity.

KEY WORDS: human immunodeficiency virus type 1, mycoplasma, $H_2O_2$ release, cytopathicity, co-infection.

Abbreviations: $H_2O_2$, hydrogen peroxide; HIV-1, human immunodeficiency virus type 1; PBMC, human peripheral blood mononuclear cells; SFU, syncytium forming unit; PHA, phytohemagglutinin from *Phaseolus vulgaris*; HRPO, horseradish peroxidase; GO, glucose oxidase from *Aspergil lus niger*; KRPG, Krebs-Ringer phosphate buffer with 5.5 mM glucose

## INTRODUCTION

Interest in reactive oxygen species, as they relate to the pathogenesis of HIV[1,2], came with the discovery that the plasma concentration of malonyldialdehyde, a byproduct of lipid peroxidation, was elevated in patients infected with HIV-1[3]. Glutathione, a thiol-containing antioxidant tripeptide that is especially important for the *in vivo* removal of intracellular $H_2O_2$, was found to be present in sub-normal levels in the plasma and lung epithelial lining fluid of HIV-infected individuals[4]. The concentrations of cysteine in plasma and glutathione in peripheral blood mononuclear cells (PBMC)[5,6] and T-lymphocyte subsets[7] of HIV-infected individuals were also reported to be low. Additionally, macaques infected with the simian immunodeficiency virus (SIV)

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displayed lower plasma thiol levels than did uninfected controls[8]. The early onset of this abnormality, one week after infection, suggested that glutathione deficiency is a sensitive, and possibly specific, biochemical marker of SIV infection *in vivo*. Since reduced glutathione may possibly be consumed through its reaction with $H_2O_2$, these findings suggest that excessive $H_2O_2$ may be produced in some cells or tissues of HIV-infected individuals.

A potential oxidant such as $H_2O_2$ can be a powerful activator of HIV-1 transcription in Jurkat T-cells[9] or in UI promonocytic cells[10], an effect mediated by NF-$\kappa$B, which is itself activated by $H_2O_2$. These *in vitro* findings indicate that HIV replication is redox-regulated, and suggest that the toxic reduced oxygen species, if released *in vivo*, may contribute to the pathogenesis of AIDS. However, direct demonstration of $H_2O_2$ release by infected T lymphocytes is still not available.

It has been suggested that mycoplasmas may play a role in AIDS pathogenesis by virtue of acting as cofactors in the progression towards AIDS[11]. For review, see ref. 11a. Initial studies led to the isolation of *Mycoplasma fermentans* from an AIDS patient suffering from Kaposi sarcoma[12]. Mycoplasmas have been isolated from the urine[13, 14] and from PBMC of HIV-infected individuals[15], and studies in patients showed a high frequency of association of antibodies to some mycoplasmas that are usually isolated from pathological sites[16,17]. However, the cofactor hypothesis remains controversial[18–20].

Mycoplasmas are facultative pathogens in man and some are known to release $H_2O_2$ and the superoxide anion radical during growth[21,22]. It is thus possible that co-infection of HIV-infected cells by mycoplasma *in-vivo* may similarly lead to the release of toxic oxygen products and thus be responsible for some of the pathologies observed in AIDS. It was the purpose of this study to establish if reduced oxygen species were produced by *in vitro* co-infecting a human T cell line with HIV-1 and mycoplasmas, and to assess the possible cytopathicity of dual infection.

Here, we describe the release $H_2O_2$ by such co-infected cells, the resultant cellular stress characterized by cytostasis, and the abrogation of these toxic effects by treatment with anti-mycoplasmal antibiotics.

## MATERIALS AND METHODS

### *Cell Lines and HIV-1 Isolates*

CEM and HUT 78 human lymphoblastoid T-cell lines were cultured in RPMI 1640 medium containing 10% fetal calf serum (FCS)[23]. HT4 LacZ-1 cells, a $CD4^+$ HeLa cell line obtained by transfection with the $\beta$-galactosidase gene of *Escherichia coli*[24], were cultured in DMEM medium plus 10% FCS. HIV-1 isolates LAI, ELI and MN were a gift from Dr. F. Barré-Sinoussi (Institut Pasteur, Paris). LAI and ELI were propagated in persistently-infected CEM or HUT 78 cells, and MN was propagated in HUT 78 cells. The infections were established at a low multiplicity[23].

### *Viral Infectivity Assay*

The titers of LAI and ELI isolates were estimated in a syncytium-forming assay on monolayers of HT4 LacZ-1 cells[24]. Briefly, duplicate wells of 24-well tissue culture plates containing confluent HT4 LacZ1 cells were inoculated with 30–100 $\mu$l aliquots of culture supernatants from infected CEM cells, or from phytohemagglutinin

(PHA)-transformed PBMC, and incubated for four days. The medium was then removed, the cells were fixed with a formaldehyde-glutaraldehyde solution, and the wells were flooded with β-galactosidase substrate to reveal the syncytia in blue. The number of syncytia was linearly correlated with the dilution of culture supernatant. The results are expressed as syncytium-forming units per ml of culture supernatant (SFU/ml).

### *PBMC Isolation*

The PBMC were obtained from HIV-antibody-negative volunteers and were isolated from sterile whole blood by centrifugation on Ficoll-Hypaque (Eurobio, France). The yield was approximately $10\times10^6$ lymphocytes from 10 ml blood. The cell concentration was adjusted to $1\times10^6$/ml and the cells were dispensed in 6-well tissue culture plates in RPMI 1640 medium plus 10% FCS and supplemented with 1 μg/ml PHA. They were grown for 2–3 days in a 5% $CO_2$/95% air atmosphere at 37°C, and then infected with 237 SFU/ml (100,000 cpm of reverse transcriptase activity/ml) of isolate ELI in the presence of 7 μg/ml of DEAE-Dextran (Sigma Chemical Corp., U.S.A.).

### *Capture Radioimmunoassay (RIA) of P24 Antigen*

Supernatant P24 antigen was estimated in a capture RIA with plates coated with the P24 antigen-specific monoclonal antibody (Mab) 25-A[25]. Bound P24 antigen was then revealed by incubating duplicate wells with $^{125}$I-labeled Mab 25-C (specific activity, $1–3\times10^6$ cpm), which recognizes a different epitope on the P24 antigen. A standard curve constructed with purified P24 antigen indicated a sensitivity of around 1 ng/ml.

### *Spectrofluorometric Assay of $H_2O_2$*

The assay was carried out according to Root *et al.*[26] with minor modifications[27]. Cell lines or PBMC growing in RPMI 1640 medium plus 10% FCS were washed twice in Krebs-Ringer phosphate buffer with 5.5 mM glucose (KRPG) at 22°C and the $H_2O_2$ released was measured using a scopoletin (6-Methoxyumbelliferone, Sigma Chemical Corp., U.S.A) fluorescence assay in which $H_2O_2$ oxidizes scopoletin to a nonfluorescent state in a reaction catalyzed by horseradish peroxidase (HRPO). The KRPG solution was prepared fresh daily. Fluorescence was measured in a Jobin Yvon 3D spectrofluorometer with an excitation wavelength of 350 nm and an emission wavelength of 460 nm. The cells ($1–4\times10^6$) were suspended in disposable "Ultra-Vu" acrylic cuvettes (Elkay, Shrewsbury, MA) in a total volume of 3 ml of KRPG containing 3.3 mM scopoletin and 1 mM $NaN_3$. The contents were mixed and allowed to equilibrate to 37°C in the thermostated cuvette holder for 10–30 min. The reaction was started by adding 15 μg HRPO, and the decrease in fluorescence emission was automatically recorded over 8 min.

The rate of $H_2O_2$ release was then calculated from the slope of fluorescence intensity decrease[26] and the values were expressed as p moles $H_2O_2/10^6$ cells/min. A control cuvette, included in each assay, contained scopoletin, HRPO and $NaN_3$, but not cells. The slope thus generated was subtracted from the values obtained for cells. A standard curve was constructed which plotted known amounts of reagent $H_2O_2$ (Prolabo, France) versus the % relative fluorescence intensity. The $H_2O_2$ concentration was linearly correlated to the fluorescence intensity over the range of 100–5,000 p moles,

and 1,000 p moles $H_2O_2$ gave a 20% drop in the relative fluorescence intensity under our assay conditions. The concentration of commercial $H_2O_2$ was assayed spectrophotometrically using E 1%, 240 nm = 43.6.

### *Mycoplasma Source and Identification*

Three species of mycoplasmas of ATCC origin were donated by Dr. D. Roulland-Dussoix (Institut Pasteur, Paris): *M. fermentans* (strain PG 18), *M. hominis* (strain PG21) and *M. pirum* (Type). They were subcultured in SP4 medium (a gift from Dr. A. Blanchard, Institut Pasteur, Paris) and stored at –80°C until use. *M. orale* was isolated from CEM cell cultures. *M. hyorhinis* was isolated from ELI culture supernatants. These contaminant mycoplasmas were grown up in SP4 medium and were all identified using specific DNA primers followed by PCR amplification at the Laboratoire des mycoplasmes, Institut Pasteur, Paris. In addition, the identity of *M. hyorhinis* was also confirmed by serological tests in the laboratory of Dr. J.G. Tully (National Institute of Allergy and Infectious Diseases, Frederick, MD).

### *Antibiotics*

The following antibiotics were used as mycoplasma removal agents in this study. BM-Cyclin (Boehringer Mannheim, Germany) is a pleuromutiline derivative (BM-Cyclin 1) plus a tetracycline derivative (BM-Cyclin 2). Cultures were treated as specified by the manufacturer by treating cells on alternate 3 and 4 days. MRA (ICN Flow, Orsay, France), a derivative of 4-oxoquinoline-3-carboxylic acid, was used at 0.05 or 0.5 $\mu$g/ml. Pefloxacine (Laboratoires Roger Bellon, France), an antibiotic used to treat Gm- bacterial infections, was used at 0.5 or 5 $\mu$g/ml.

### *Enzymes*

Glucose oxidase (GO) (35 U/mg) from *Aspergillus niger* was from Biozyme, UK. Beef liver catalase (100,000 U/mg) was from Boehringer Mannheim, Germany. HRPO (1,000 U/mg in the ABTS assay[28]) was from Sigma Chemical Corp. U.S.A.

## RESULTS

### *Release of $H_2O_2$ from HIV-1-infected CEM and HUT 78 Cells Detected by Scopoletin Fluorescence Quenching*

The release of $H_2O_2$ from HIV-1-infected cells was estimated spectrofluorometrically using the scopoletin fluorescence quenching technique. Figure 1 shows a typical assay performed on persistently ELI-infected HUT 78 cells and control uninfected HUT 78 cells. In this experiment the infected cells released 23 p moles $H_2O_2/10^6$ cells/min, and uninfected HUT 78 cells released <1 p mole $H_2O_2/10^6$ cells/min. A mixture of all components except cells consistently gave a background decrease in fluorescence intensity equivalent to 14 p moles/min similar to that seen with uninfected HUT 78 cells. When this background was subtracted, a net release of as little as 1 p mole $H_2O_2/10^6$ cells/min could be detected, and the curve which plotted cell numbers versus $H_2O_2$ release passed through 0 (Fig. 2). Under our experimental conditions, $H_2O_2$ release remained linear with cell numbers of up to $4\times10^6$/cuvette (Fig. 2).

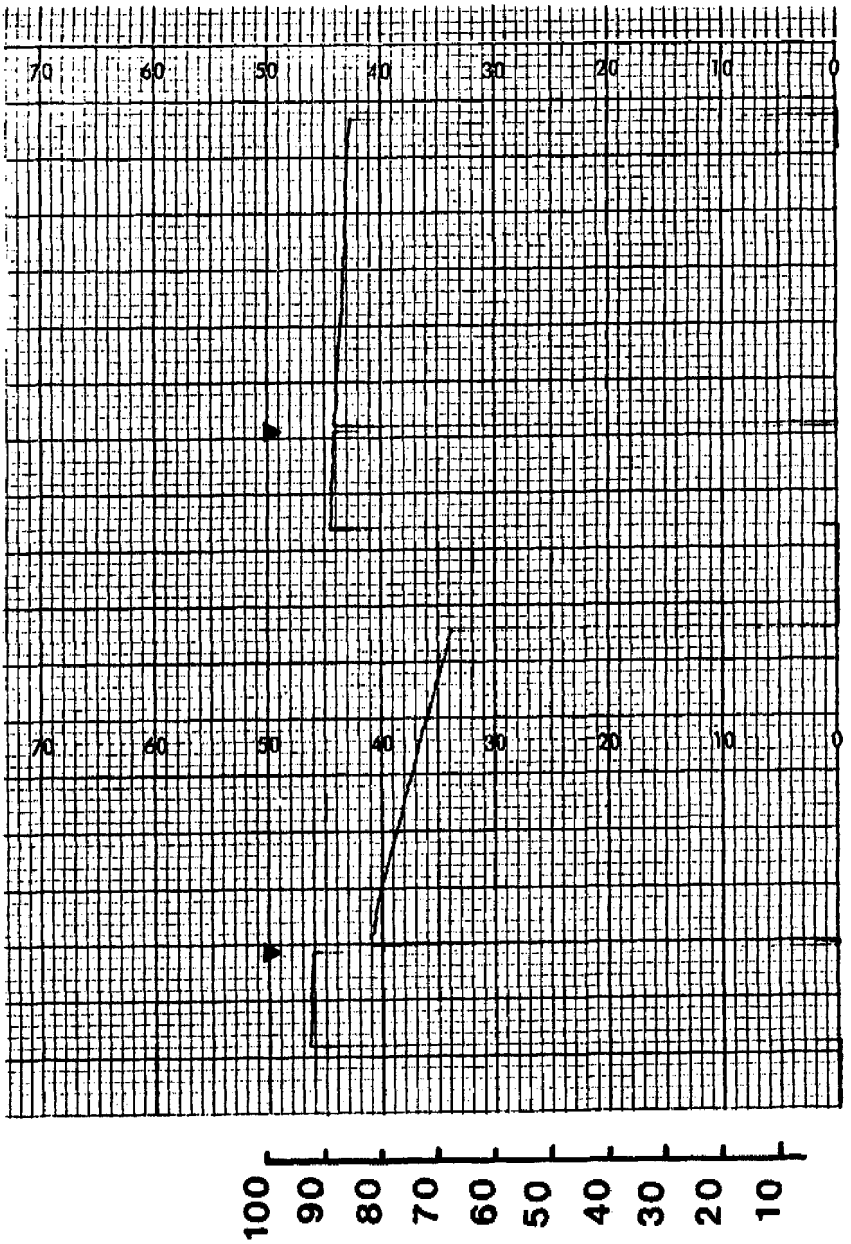


FIGURE 1 Spectrofluorometric determination of $H_2O_2$ release by persistently ELI-infected HUT 78 cells (left hand tracing), and control uninfected HUT 78 cells (right hand tracing). Cuvettes contained $2.88\times10^6$ ELI-infected cells or $4.4\times10^6$ control uninfected HUT 78 cells in a total volume of 3 ml KRPG. The oxidation reaction was started by the addition of 15 $\mu$g HRPO (arrowhead). The smallest square represents 20 sec. of recording time.

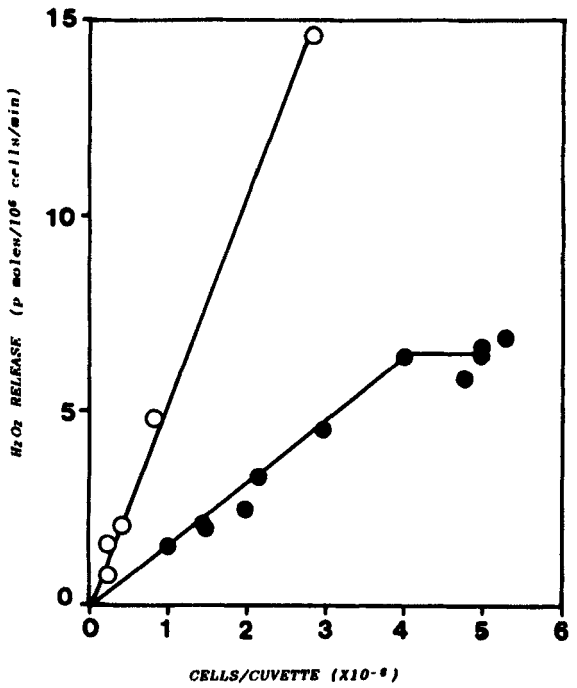


FIGURE 2 Plot of cell numbers versus rate of $H_2O_2$ release. (○), persistently ELI-infected HUT 78 cells; (●), persistently LAI-infected HUT 78 cells. Each point represents a single assay.

The oxidation of scopoletin was strongly catalase-sensitive confirming that $H_2O_2$ was being measured. The addition of 4333 U catalase to cuvettes containing ELI-infected CEM cells 5 min after HRPO had been added produced an immediate and pronounced flattening of the slope (data not shown).

Table 1 summarizes the $H_2O_2$ release data gathered over a period of 8 months. Persistently-infected CEM to HUT 78 T cells and acutely-infected human PBMC with LAI, ELI or MN released mean values of 16–84 p moles $H_2O_2/10^6$ cells/min. Uninfected CEM cells released traces of 1 p mole $H_2O_2/10^6$ cells/min above background, whereas HUT 78 cells were negative. Trace levels of 1 p mole/$10^6$ cells/min or less were found in seven transformed HIV-1-negative cell lines. We failed to detect the superoxide anion in any of the HIV-1-infected cells (data not shown).

### *Time Kinetics of $H_2O_2$ Appearance in LAI Acutely-infected CEM Cells*

CEM cells were acutely infected with LAI in order to determine the time at which $H_2O_2$ and infectious virus production began. As shown in Figure 3, as early as 3 days after infection, $H_2O_2$ release was easily detectable with a mean value of around 5 p moles/$10^6$ cells/min, whereas P24 antigen or infectious virus production were not yet observed. $H_2O_2$ release gradually increased thereafter to reach peaks of approximately 45 pmoles/$10^6$ cells/min on days 21 and 39.

TABLE 1
Rates of $H_2O_2$ release by HIV-1-infected and control cells

| Cell type | Infecting HIV-1 | p moles $H_2O_2/10^6$cells/min in (n) samples |
|---|---|---|
| CEM | LAI[a] | 33 ± 29 (26) |
| CEM | ELI[a] | 84 ± 73 (12) |
| HUT 78 | MN[a] | 20 ± 13 (9) |
| PBMC[b] | ELI | 16 ± 5 (6) |
| CEM | | 1 ± 0.80 (12) |
| HUT78 | | 0 (8) |
| PBMC[c] | | 2 ± 0 (2) |
| SU-1[d] | | 1 (1) |
| L1210 | | 1 (1) |
| BCL-1 | | 1 (1) |
| X63 | | 1 (1) |
| 8E5 | | 0 (1) |
| SU4–50 | | 0 (1) |
| 097 | | 0 (1) |

[a] Persistently infected cell lines.
[b] PBMCs were acutely infected with 3.3 SFU/ml ELI 3 days after stimulation with PHA and tested on day 14.
[c] Tested on day 3 after stimulation with PHA.
[d] SU-1 and SU4–50 are human B lymphoma lines; L1210 and BCL-1 are mouse B lymphoma lines; X63 is a mouse myeloma line; 8E5 is human T-lymphoma line; 097 is a mouse B-hybridoma. Values are means and S.E.M.

### *Treatment with Anti-mycoplasmal Antibiotics Reduces Levels of $H_2O_2$ in HIV-1-infected CEM Cells and PBMC*

Treatment of persistently HIV-1-infected CEM cells with anti-mycoplasmal antibiotics, described in Materials and Methods section, resulted in a sharp fall in $H_2O_2$ release which

TABLE 2
Effect of anti-mycoplasmal antibiotics on $H_2O_2$ release and virus production in HIV-1-infected CEM cells and PBMC

| Cell type (HIV-1) | Antibiotic[a] | p mole $H_2O_2$/ $10^6$ cells/min | SFU/ml |
|---|---|---|---|
| CEM (LAI)[c] | none | 72 ± 42 | 4,700 ± 300 (4) |
| CEM (LAI) | BM | 1 ± 1[b] | 5,080 ± 620 (5)[b] |
| CEM (ELI)[c] | none | 79 ± 79 | 2,250 ± 250 (4) |
| CEM (ELI) | BM | 2 ± 1[b] | 3,350 ± 50 (4)[b] |
| CEM (ELI) | MRA | 2 ± 2[b] | 3,750 ± 350 (4)[b] |
| CEM (ELI) | Pef | 3 ± 2[b] | 1,800 ± 200 (4)[b] |
| PBMC (ELI)[c] | none | 24 ± 3 | 1,955 ± 95 (4) |
| PBMC (ELI/BM)[d] | | 1 ± 1 | 1,940 ± 380 (5) |
| PBMC (ELI/MRA)[d] | | 3 ± 2 | 1,890 ± 270 (2) |
| PBMC (ELI/Pef)[d] | | 2 ± 1 | 1,865 ± 385 (2) |

[a] BM, BM-Cyclin (10 μg/ml BM-Cyclin 1 for 3 days alternating with 5 μg/ml BM-Cyclin 2 for 4 days, treatment time was 21 days); MRA, 4-oxoquinoline-3-carboxylic acid (0.5 μg/ml), treatment time was one week; Pef, Pefloxacine (5 μg/ml), treatment time was one week.
[b] Estimated on days 3–7 of antibiotic treatment.
[c] CEM (LAI) and CEM (ELI) were from persistently-infected cultures; PBMCs were acutely infected with 3.3 SFU/ml of ELI 2–3 days after stimulation with PHA.
[d] PBMCs were infected with 3.3 SFU/ml of ELI suspensions that came from persistently infected CEM cell cultures treated with anti-mycoplasmal antibiotics in order to obtain ELI free of mycoplasma. The treatment times and doses of antibiotics were as above. $H_2O_2$ release was estimated on day 13 and syncytia were assayed from day 14 supernatants.
The number of independent experiments is indicated in parentheses; each value is the mean and S.E.M.

dropped from around 70 p moles/$10^6$ cells/min to 1–3 p mole/$10^6$ cells/min (Table 2). After 9–10 days of antibiotic treatment, $H_2O_2$ was no longer detectable (<1 p mole/$10^6$ cells/min) and remained so for up to 112 days reflecting the effective removal of mycoplasma, which was also verified by the absence of growth in SP4 medium.

The possibility that inability to detect $H_2O_2$ might be due to a direct interaction of cell-accumulated drugs with $H_2O_2$, or to inhibition of the $H_2O_2$-producing enzyme system was ruled out in separate experiments. First, neither of the drugs, except for BM-Cyclin 2, inhibited an $H_2O_2$ flux generated by glucose oxidase/glucose. The only inhibition observed by BM-Cyclin 2 was possibly due to some interference with HRPO activity as indicated by still other tests in which HRPO was preincubated with the drug. Second, neither of the drugs caused a significant inhibition of superoxide release from phorbol myristate acetate (PMA)-stimulated human polymorphonuclear leukocytes (PMN) in a cytochrome C reduction assay. The PMN had been preincubated for 20 min before being tested, and the assays included a four-fold greater concentration of the antibiotics than that added to the cultures (data not shown). Therefore, the abrogation of $H_2O_2$ release by the antibiotics was most likely due to mycoplasmal death rather than artefactual.

Culture supernatants of ELI contained *M. hyorhinis*, and supernatants of LAI contained *M. hyorhinis*, *M. fermentans* and *M. orale*. *M. orale* was also found as a contaminant in CEM cells.

Treatment of HIV-1-infected cell lines with these anti-mycoplasmal antibiotics resulted in the following additional observations. First, the antibiotics had no significant effect on the infectious virus titer (Table 2), even when estimated 100 days after treatment (data not shown). Second, there was a notable improvement in cell viability that became evident as early as 3 days after antibiotic treatment. Cell mortality of long-term persistently HIV-1-infected cultures, usually 20–40%, fell rapidly and by one week of antibiotic treatment viability returned to nearly 100%, comparable to uninfected CEM or HUT 78 cells.

$H_2O_2$ release was also studied in normal PHA-stimulated human PBMCs. PBMCs were acutely infected with ELI culture supernatants which came from CEM cultures treated with anti-mycoplasmal antibiotics in order to eliminate infectious mycoplasmas. Such supernatants, however, did not prove to be entirely free of mycoplasmas (see Legend to Table 3). As shown in Table 2, removal of mycoplasmas by either of BM-Cyclin, Pefloxacine or MRA antibiotics, resulted in much reduced levels of $H_2O_2$ release (1–3 p mole/$10^6$ cells/min) whereas control PBMCs infected with mycoplasma-contaminated ELI supernatants released as much as 24 p moles $H_2O_2$/$10^6$ cells/min. Furthermore, there was no significant effect on the HIV-1 titer following antibiotic treatment (Table 2).

### *Co-infected, but not Singly-infected CEM Cells Release $H_2O_2$*

The above experiments suggested that $H_2O_2$ release in persistently-infected cultures was due solely to mycoplasmal infection, since their elimination by anti-mycoplasmal antibiotics strongly decreased $H_2O_2$ release. However, the possible contribution of co-infecting HIV to mycoplasmal $H_2O_2$ production and release could not be determined by this approach.

Separate CEM cultures were therefore acutely infected with ELI culture supernatants (from BM-Cyclin-treated cultures, see Legend to Table 3) together with one of five species of mycoplasmas, with the mycoplasmas alone, or with virus alone. Table 3 shows one of seven similar experiments. Co-infection with both ELI and mycoplasmas

TABLE 3
$H_2O_2$ release from CEM cells acutely infected with ELI and mycoplasmas

| Infectious agent | $H_2O_2$ release (p moles/$10^6$ cells/min) | P | cells/dish ($\times 10^{-6}$) | P |
|---|---|---|---|---|
| ELI[a] & *M. hyorhinis*[b] | 10.7 ± 6.3[d] | 0.02 | 3.3 ± 2.1 | 0.04 |
| ELI & *M. orale* | 3.6 ± 0.4 | 0.0008 | 3.9 ± 0.5 | 0.012 |
| ELI & *M. fermentans* | 10.2 ± 8.2 | 0.05 | 2.6 ± 1 | <0.0005 |
| ELI & *M. hominis* | 6.7 ± 4.7 | 0.04 | 3.6 ± 1.5 | 0.01 |
| ELI & *M. pirum* | 6.2 ± 2.1 | 0.003 | 3.1 ± 0.8 | >0.05 |
| *M. hyorhinis* | 2 ± 1 | | 6.5 ± 1.4 | |
| *M. orale* | 1 ± 0 | | 6.3 ± 1.1 | |
| *M. fermentans* | 0 | | 8.2 ± 0.4 | |
| *M. hominis* | 0 | | 8.6 ± 1.4 | |
| *M. pirum* | 2 ± 1 | | 4.7 ± 1.9 | |
| none | 1.5 ± 0.5 | | 8.5 ± 1.6 | |
| ELI | 3 ± 3.1[e] | | 5.6 ± 0.9 | |
| ELI & *M. hyorhinis*[c] | 45 ± 11 | | 1.7 ± 0.2 | |

[a] For these experiments ELI was obtained from persistently infected CEM cells treated for 21 days with BM-Cyclin in order to obtain virus suspensions free of mycoplasmas. Culture supernatants were then collected starting on day 21. CEM cells were infected with 33 SFUs ELI /5 ml culture dish.
[b] 50 $\mu$l of mycoplasma suspension in SP4 medium/dish was used for infection.
[c] Persistently infected HUT 78 cells. Mean and S.E.M. of 9 measurements.
[d] Values are means and S.E.M. of 3–4 measurements on days 4, 8, 12 and 16 after infection. P values compare ELI plus mycoplasmas versus mycoplasmas alone.
[e] $H_2O_2$ release was undetectable on days 4 and 8, was 5 p moles/$10^6$ cells/min on day 12, and 7 p moles/$10^6$ cells/min on day 16.

gave an average $H_2O_2$ release of 4–11 p moles/$10^6$ cells/min, but no increase in $H_2O_2$ release was found after infection with ELI alone. There was, however, a delayed $H_2O_2$ release found with ELI alone on days 12 and 16 (see Legend to Table 3) which is thought to be due to the growth of residual *M. hyorhinis* not fully eradicated by antibiotic treatment. This is supported by data that always showed an early (3–4 days, see Fig. 3a) release of $H_2O_2$ from CEM cells infected with ELI supernatants that came from cultures not treated with antibiotics. Furthermore, CEM cultures infected with the mycoplasmas alone produced little or no $H_2O_2$ (Table 3). Even a five-fold increase of the mycoplasmal inoculum did not produce any significant increase of $H_2O_2$ (data not shown). Thus, only the simultaneous presence of HIV-1 and the various mycoplasmas appeared to result in a detectable $H_2O_2$ release from CEM cells.

Additionally, cytostasis occurred in co-infected CEM cultures but not in cells infected with mycoplasma or virus alone (Table 3). The growth of co-infected cultures was more severely inhibited (2.6–3.9×$10^6$ cells/dish) than was the growth of mycoplasma-infected cultures (4.7–8.6×$10^6$ cells/dish) (Table 3). Hence, cytostasis appeared to be a specific outcome of HIV/mycoplasma co-infection.

### *Inhibition of LAI Replication by Catalase*

A recent report showed that $H_2O_2$ can activate HIV-1 expression and replication *in vitro*[9] suggesting that endogenous $H_2O_2$ might be needed for optimal HIV replication. However, as stated above, in our hands the replication of HIV-1 was not altered by reduction of $H_2O_2$ levels to <1 p mole/$10^6$ cells/min. Since $H_2O_2$ is freely diffusible across the cell membrane, its intracellular production would have been detected.

Nevertheless, in order to investigate the possible presence of still lower amounts of $H_2O_2$ we tested the enzyme catalase for its effect on HIV replication. Addition of catalase

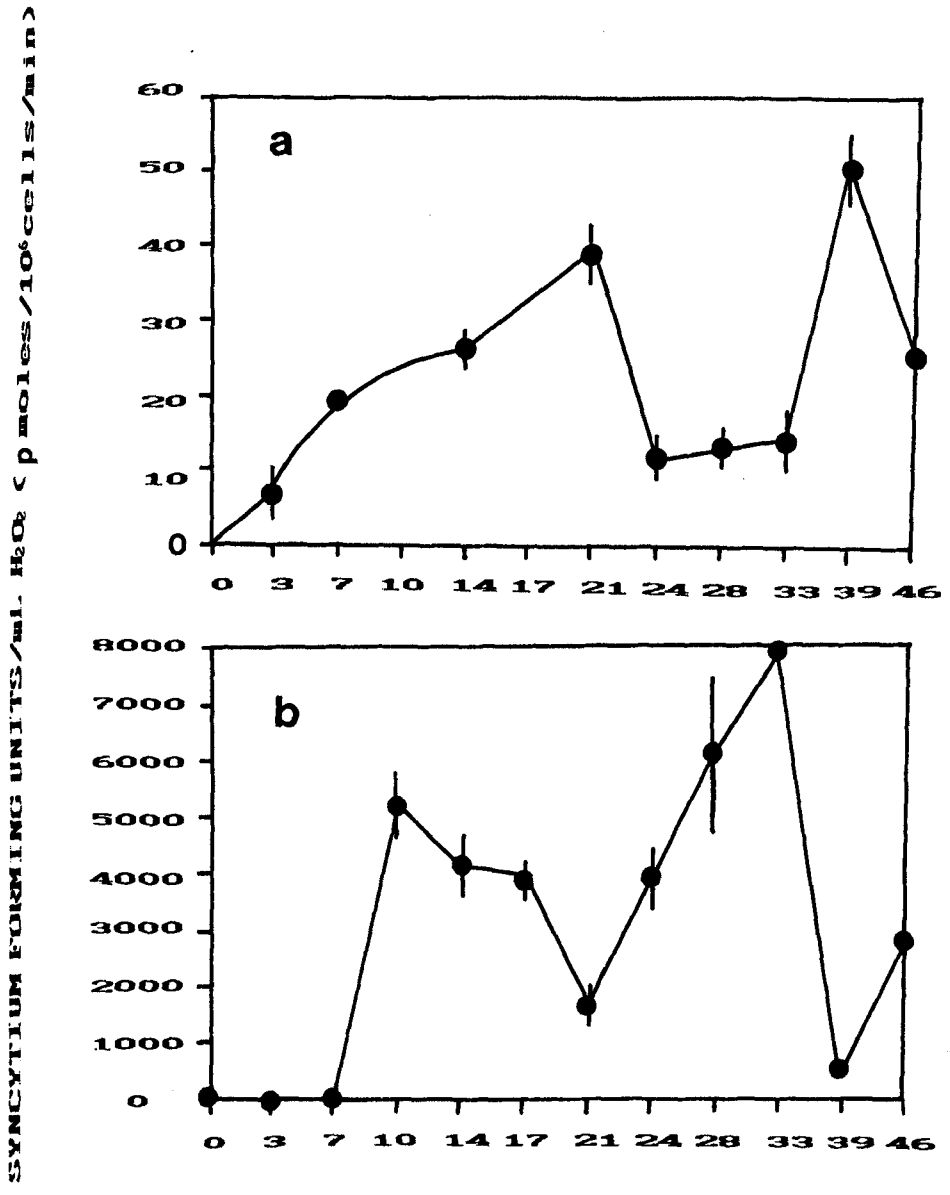


FIGURE 3 Acute infection of CEM cells with isolate LAI showing $H_2O_2$ release (a), infectious virus titer (b), and P24 antigen levels (c). Triplicate cultures were infected on day 0 with 28 SFU/ml LAI. Each data point is the mean and S.E.M. of single in (a), or duplicate determinations in (b) and (c).

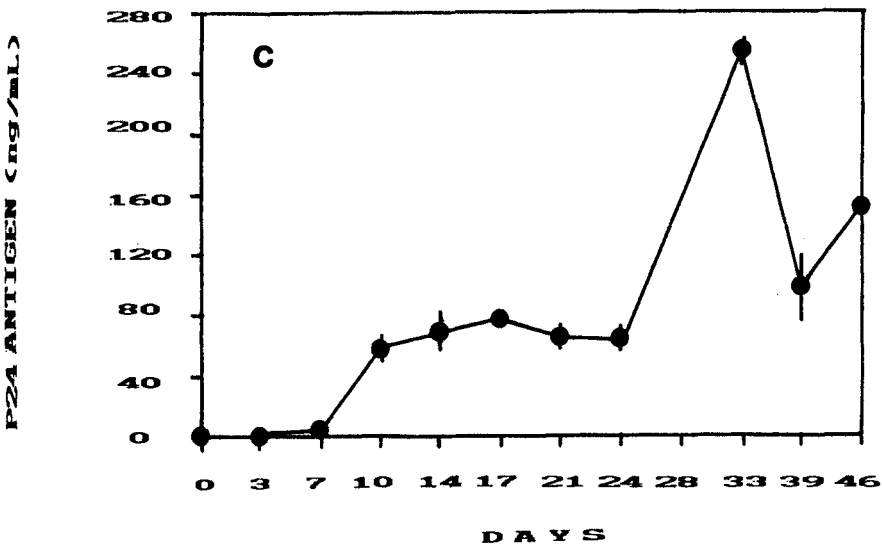


FIGURE 3 *continued*

to cultures of persistently LAI-infected CEM cells, that were also mycoplasma-contaminated, strongly inhibited LAI replication in a dose-dependent fashion (Fig. 4). There was no effect on cell viability at the doses tested (data not shown).

As little as 10 U/ml catalase produced nearly 50% inhibition of SFUs (Fig. 4), suggesting a high sensitivity of HIV replication to inhibition by catalase. Inactivation of catalase by heat destroyed its inhibitory effect, consistent with the enzymatic nature of the inhibition. Supplementation of cultures with 0.3 mU/ml of GO, to generate $H_2O_2$, partially reversed the inhibitory effect of 5,000 U/ml of catalase, (data not shown) suggesting that catalase was acting by destroying peroxide.

## DISCUSSION

This study shows that dual acute infection of the human CEM T lymphocytes with HIV-1 and any one of five mycoplasma species leads to the release of easily measurable levels of $H_2O_2$ and to cytopathicity. Although mycoplasmas are known to produce $H_2O_2$[21,22], $H_2O_2$ was not detected in CEM cells infected with mycoplasmas alone (see Table 3). However, this $H_2O_2$ was most likely of mycoplasmal origin since co-infected cells treated with any one of three different anti-mycoplasmal antibiotics produced a sharp fall of $H_2O_2$ release without affecting the HIV-1 titers (see Table 2). Such antibiotic-treated cultures displayed nearly 100% viability, and neither significant cell mortality, cytostasis or the release of $H_2O_2$ were detected. Also, infection of human PBMCs with ELI culture supernatants treated with anti-mycoplasmal antibiotics, in order to remove infectious mycoplasmas, showed a much reduced $H_2O_2$ release relative to untreated ELI culture supernatants (see Table 2).

Attempts to clarify the contribution of HIV-1 and mycoplasmas to $H_2O_2$ release were made by separately infecting CEM cells with these organisms (see Table 3). Only by such an experimental approach could we show that both virus and any one of five mycoplasma species must simultaneously infect CEM cells, and somehow interact, to

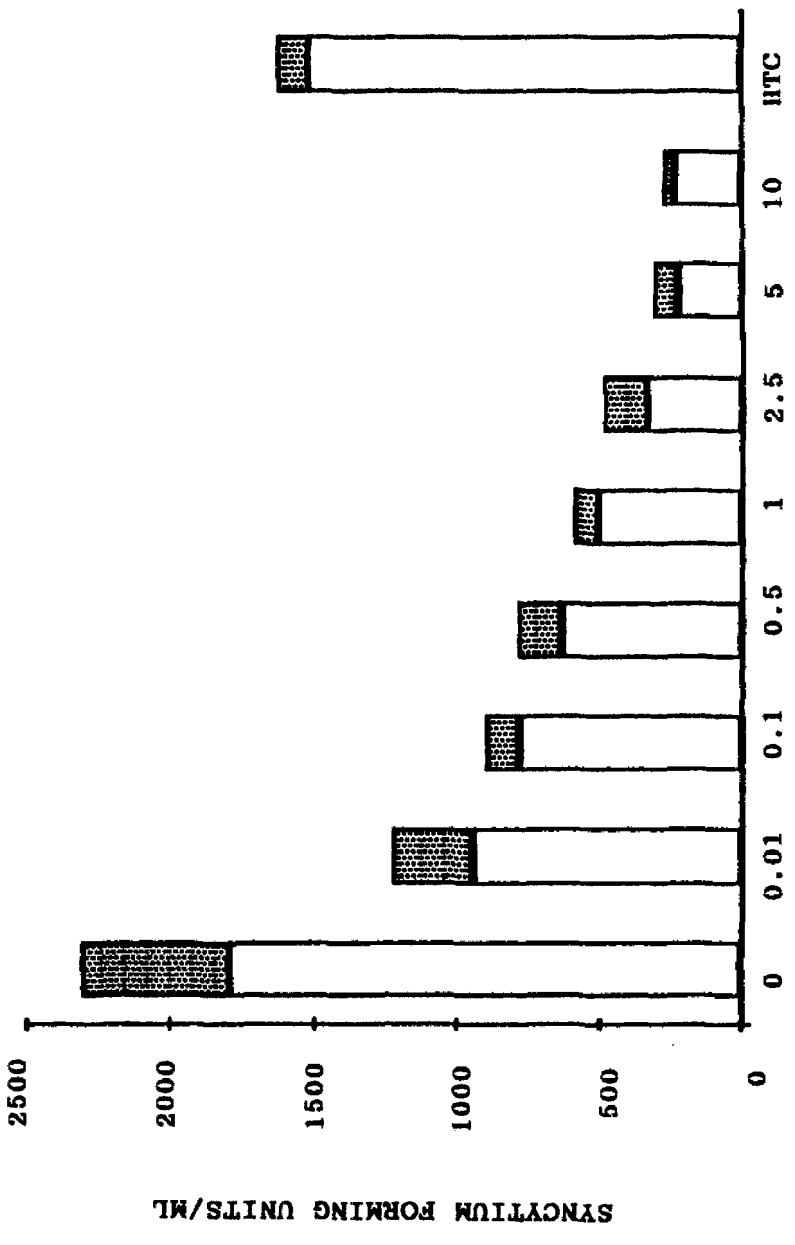


FIGURE 4 Inhibition of LAI replication by catalase. Increasing amounts of catalase were added to cultures of persistently-infected CEM cells on day 0. The virus titer (SFU/ml) was determined on day 4 supernantants. The bars represent means and S.E.M. (stipled areas over the bars) of four separate experiments. HTC, 10 kU/ml of heat-treated catalase (100°C, 15 min). P = 0.80, no catalase added versus HTC added.

allow measurable $H_2O_2$ release. Thus, while mycoplasmal $H_2O_2$ release appeared to depend on active HIV-1 replication, this was observed only with the CEM cell line. With HUT 78 cells this interaction was not seen (unpublished data). In the present study, therefore, the CEM cell line was most suitable for studying HIV-1/mycoplasma co-infection in regards to the release of $H_2O_2$.

The previous observation that HIV-1-infected CEM cells and PBMCs were highly susceptible to *in vitro* lysis by human myeleperoxidase[25] now finds an explanation by the present demonstration of $H_2O_2$ release by these co-infected cells. Even as low levels as 5 p moles $H_2O_2/10^6$ cells/min, which serves a substrate for this enzyme, sufficed for effective cell lysis (unpublished data).

In view of the potentially toxic nature of $H_2O_2$, the present data suggest that cell mortality and cytostasis might have originated largely from $H_2O_2$ produced in co-infected cells. This study confirms previous work[15,29] that also incriminated mycoplasmas as being mainly responsible for an *in vitro*-generated cytopathic effect of HIV-1/mycoplasma co-infected cultures. However the possible underlying biochemical events responsible for cytopathicity have not been investigated in those reports.

Co-infected cells could produce excessive $H_2O_2$ in several ways. First, HIV-1 may interact with mycoplasmas by stimulating growth. Examples of such an interaction between HIV-1 and a co-infecting microorganism, yielding increased HIV-1 and co-infector multiplication, have been recently described[30–32]. Second, any one of several known mammalian oxidases[33] may become activated as a result of co-infection, or on the contrary cellular antioxidative defences may be down-regulated. Recently, a HIV-1-associated oxidative stress was described *in vitro* as being due to down-regulation of the anti-oxidative enzymes catalase[34] or glutathione peroxidase[35], both of which specifically destroy $H_2O_2$.

The scopoletin fluorescence quenching assay used to estimate $H_2O_2$ has been used previously to quantify the rates of $H_2O_2$ release from various mammalian cell types. Some human tumor cells lines release as much as 830 p moles $H_2O_2/10^6$ cells/min[36], and *Staphylococcus aureus*-stimulated PMN release a mean of 114 p moles $H_2O_2/10^6$ cells/min[26]. PMA-stimulated PMN, on the other hand, may release up to 2,300 p moles $H_2O_2/10^6$ cells/min[36]. Therefore, the release of 4–84 p moles $H_2O_2/10^6$ cells/min, found in the present study, is in the lower range of $H_2O_2$ release, but it may be important physiopathologically if it were to occur *in vivo*.

The potentially toxic nature of $H_2O_2$ is indicated by its capacity to interact with the superoxide anion in the presence of free iron, in the Fenton reaction, to give rise to hydroxyl radicals, a highly toxic and damaging oxygen species that can initiate peroxidation of cellular lipids[37]. Similarly, $H_2O_2$ can oxidize chloride ions to form hypochlorous acid which can then react with superoxide anions to generate the hydroxyl radical, in a reaction claimed to proceed at yet faster rates[38]. There are several ways by which uncontrolled release of $H_2O_2$ in HIV-infected patients may lead to an exacerbation of observed pathologies. $H_2O_2$ may contribute to the steady decline of CD4 lymphocytes which die by apoptosis[39], as observed in immunodeficient individuals[40]. Recent findings suggest that apoptosis may be initiated by $H_2O_2$, at least in some cell models[34,41]. $H_2O_2$ may also cause oxidative damage to DNA by indirectly generating hydroxyl radicals[42] and thus favor the emergence of HIV-1 mutants that have been described in HIV-1-infected individuals[43]. Such mutants can then escape neutralization by antibodies *in vivo* and infect new cells.

Moreover, a key role of $H_2O_2$ as a stress factor in HIV-1 replication has recently been described in which reagent $H_2O_2$ can act directly as an HIV-1 inducer by activating the cellular transcription factor NF-$\kappa$B in Jurkat T-cells[9]. Work from several

laboratories now indicates that NF-$\kappa$B is crucially important in HIV-1 replication and is redox regulated[44] underlining the possible importance of $H_2O_2$ production for the initiation of HIV-1 replication *in vitro*.

The present study confirms the importance of $H_2O_2$ in HIV-1 replication, and furthermore suggests that the $H_2O_2$ concentrations needed for optimal HIV-1 replication may lie in a low range probably of <1 p mole/$10^6$ cells/min. Only by growing CEM cells in the presence of catalase was it possible to significantly reduce the HIV-1 titer (see Fig. 4). Catalase probably destroyed all of the mycoplasma-generated $H_2O_2$, which should be readily accessible to the enzyme because mycoplasma grow on the cell wall. In addition, catalase presumably also destroyed traces of $H_2O_2$ of endogenous origin, in spite of its large size preventing ready intracellular penetration. The HIV-1-inhibitory activity of catalase is compatible with reports showing that other antioxidant molecules with known capacity to consume $H_2O_2$, such as N-acetylcysteine or glutathione, can inhibit HIV-1 expression[45,46].

On the other hand, *in vivo* HIV-1 replication might be compatible with the presence of somewhat larger amounts of $H_2O_2$ ($\approx$ 100 p moles/$10^6$ cells/min) as might occur during an inflammatory response where activated phagocytic leukocytes release $H_2O_2$. This is supported by the present *in vitro* model in which levels of 40–50 p moles $H_2O_2$/$10^6$ cells/min, in acutely-infected CEM cells (see Fig. 3), or up to a mean of 84 p moles/$10^6$ cells/min in persistently-infected cells (see Table 1) did not impair significantly infectious HIV-1 production.

Some of the mycoplasma species which are toxic in co-infected CEM cells, such as *M. fermentans* and *M. pirum* have also been isolated from patients suffering from AIDS[15,29]. Those strains came from pathological conditions, and are undoubtedly more virulent than laboratory strains or tissue culture contaminants. However, the strains studied here are involved in $H_2O_2$ release or cytostasis, both of which may be considered as markers of virulence. This suggests that the present *in vitro* model may be relevant to pathogenic processes prevalent in HIV-infected individuals. Finally, the interaction between HIV-1 and mycoplasmas in producing cytotoxicity and $H_2O_2$ release in the CEM cells is compatible with the proposed cofactor role of mycoplasmas in the development of AIDS[11].

## *Acknowledgments*

We thank Dr. Axel Périanin (Laboratoire d'Hématologie, INSERM U294, CHU Xavier Bichat, Paris, France) for performing the oxygen burst assays with human PMN and for helpful discussion. This work was supported by A.N.R.S. grant 95.8919, and in part by the C.N.R.S. and the Université de Paris.

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Accepted by Professor J.M.C. Gutteridge