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Virucidal Effect of Myeloperoxidase on Human Immunodeficiency Virus Type 1-Infected T Cells

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Myeloperoxidase is virucidal to human immunodeficiency virus type 1 (HIV-1) in the persistently infected CEM human T-cell line or in acutely infected human peripheral blood mononuclear cells, as judged by viral infectivity and P24 radioimmunoassay. HIV-1 was specifically inactivated by low doses of the human myeloperoxidase (1.4 to 14.3 mU/ml) and the cells were spared. A higher enzyme concentration (143 mU/m) was cytotoxic, but uninfected CEM cells and normal lymphocytes were resistant to ≥143 mU of myeloperoxidase per ml. The enzyme was virucidal with the Cl^- present in medium and did not require exogenous H_2O_2. Catalase, an antioxidant enzyme, partially inhibited the virucidal effect of myeloperoxidase. Hence, the H_2O_2 probably came from the HIV-infected cells themselves. These in vitro findings indicate that the myeloperoxidase system is capable of inactivating HIV-1 of infected cells.

Neutrophils and monocytes of several mammalian species contain in their primary lysosomal granules the heme enzyme myeloperoxidase (MPO). This enzyme and other mammalian peroxidases, such as the lactoperoxidase (LPO) in milk and saliva and eosinophil peroxidase in eosinophils, form a powerful microbicidal system which is effective against a number of microorganisms, including viruses (5, 13, 14). Stimulation of neutrophils results in a respiratory burst with the metabolic production of H_2O_2 (2). In its presence, MPO oxidizes Cl^- to produce hypochlorous acid, which has strong oxidant properties (10, 15).

Individuals infected with the human immunodeficiency virus (HIV) were recently reported to have depressed glutathione levels in the plasma and lungs (3). Since glutathione, the most abundant intracellular thiol, plays a key role as an antioxidant, oxidant stress may now be considered a factor in the pathogenesis of AIDS (9).

We (25) and others (5, 13, 14, 18) have recently shown that human MPO or bovine LPO, together with glucose oxidase (GO) and a halide, are virucidal to cell-free HIV type 1 (HIV-1). We have now extended our studies to show that these components are virucidal in HIV-1-infected CEM T cells as well as in HIV-1-infected T cells from phytohemagglutinin (PHA) stimulated peripheral blood mononuclear cells (PBMC).

MATERIALS AND METHODS

Cell lines and HIV isolates. CEM cells, a human lymphoblastoid T-cell line, were cultured in RPMI 1640 medium containing 10% fetal calf serum (FCS), as described previously (25). HT4 LacZ-1 cells, a $CD4^+$ HeLa cell line obtained by transfection with the β-galactosidase gene of *Escherichia coli* (20), were cultured in Dulbecco modified Eagle medium plus 10% FCS. HIV-1 isolates LAI and ELI were a gift from F. Barré-Sinoussi (Institut Pasteur, Paris). The isolates were propagated in persistently infected CEM cells with infection established at a low multiplicity (25).

Viral infectivity assay. The titers of LAI and ELI isolates were estimated in a syncytium-forming assay on monolayers of HT4 LacZ-1 cells (20), modified as described elsewhere (25). The results are expressed as syncytium-forming units per milliliter of culture supernatant or per 10^4 cells.

Capture radioimmunoassay of P24 antigen. Supernatant P24 antigen was estimated in a solid-phase capture radioimmunoassay on flexible 96-well plates (Falcon, no. 3911), using murine monoclonal antibodies (MAb) raised against purified P24 antigen from the HIV isolate LAI (a gift from F. Traincard, Institut Pasteur). The plates were coated with capture MAb 25-A, and MAb 25-C was the detecting antibody which recognized a different epitope on the P24 antigen. MAb 25-C was radiolabeled with ^{125}I to a specific activity of 1×10^6 to 3×10^6 cpm/μg. A standard curve was constructed with purified LAI P24 antigen (a gift from H. V. J. Kolbe, Transgène, Strasbourg, France). All assays were done in duplicate; the sensitivity was around 1 ng/ml. The results are expressed as nanograms of P24 antigen per milliliter of culture supernatant.

Inactivation of supernatant cell-released and cell-associated virus. Persistently infected or control uninfected CEM cells were seeded at 1×10^5 cells per ml in duplicate wells of 24-well tissue culture plates. Human MPO (1.43 to 286 mU/ml) or LPO in 20 μl of RPMI 1640 was added to the medium on day 0. After 4 days, the cells were pelleted by centrifugation and the supernatants were collected in order to assay for residual infectious virus (syncytium-forming assay) or P24 antigen (capture radioimmunoassay) as described above. The sedimented cells were washed twice with fresh culture medium, and their viability was determined in a tetrazolium dye colorimetric assay (1). In some experiments, 10,000 twice-washed HIV-1-infected CEM cells were assayed for their content of residual infectious virus in the assay described above.

Infection of human PBMC with HIV-1. PBMC were ob-

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tained from HIV-antibody-negative volunteers by Ficoll-Hypaque separation (Eurobio Laboratories, Paris, France). A total of 3×10^6 cells in RPMI 1640 plus 10% FCS were placed in six-well tissue culture plates, and PHA was added (1 μg/ml). The cells were infected with HIV isolate ELI (100,000 cpm of reverse transcriptase activity per ml) and 7 μg of DEAE-dextran per ml (Sigma Chemical Co.) was added on day 2 or 3, and the cultures were replenished with 3×10^6 freshly prepared PHA-stimulated PBMC per well together with interleukin 2 (20 U/ml) (Sigma Chemical Co.) on days 5 or 7 and 12 or 14. The cells were transferred in duplicate to 24-well tissue culture plates at 10^6 cells per ml on day 14 or 16, and various amounts (1.43 to 143 mU/ml) of MPO were added. Residual supernatant virus and cellular viability were assessed 4 days later.

Enzymes. LPO from bovine milk (M_r, 78,000; 164 U/mg) was from Biozyme (Gwent, United Kingdom). MPO purified from the HL-60 promyelocytic cell line of human origin (4) (M_r, 155,000; 19 U/mg) was a gift from T. Bringman (Ideon Corp., Redwood City, Calif.). Human recombinant MPO (M_r, 84,000; 28 U/mg) was purified from the culture supernatants of transfected Chinese hamster ovary cells (17). GO from *Aspergillus niger* (M_r, 160,000; 35 U/mg) was from Biozyme. Catalase from beef liver (M_r, 160,000; 100,000 U/mg) was from Boehringer (Mannheim, Federal Republic of Germany).

The activities of the peroxidases were estimated spectrophotometrically by using 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt as the chromogenic substrate (7). In our earlier assay (25), 0.07 U of peroxidase was equivalent to 1 U of peroxidase in the present assay. There was no loss of MPO activity after incubation for 3 days at 37°C in RPMI 1640–FCS; 90.3% of the starting LPO activity remained after 6 days. GO activity was estimated spectrophotometrically, using *O*-dianisidine as the chromogenic substrate (12). Catalase activity was estimated spectrophotometrically by the decomposition of H_2O_2 (24). Its half-life in culture was 13.6 h. In some experiments, catalase was added to cultures every 1 or every 2 days; this produced no significant difference from the standard single addition on day 0.

Statistics. Statistical analyses were done with the Student *t* test.

RESULTS

Virucidal properties of MPO in HIV-1-infected CEM cells. Figure 1 illustrates the virucidal effect of MPO against HIV-1 in persistently infected CEM cells. A low concentration of MPO (1.43 mU/ml) inactivated nearly 40% of the cell-released supernatant virus, while a higher dose (14.3 mU/ml) inactivated up to 80% of the virus (top left-hand panel in Fig. 1).

These MPO concentrations were selectively toxic against the virus since they did not affect cell viability (bottom left-hand panel in Fig. 1). MPO was cytotoxic to infected CEM cells only at 143 mU/ml; this concentration both killed the cells and inactivated all the cell-released virus. Similar results were obtained in another experiment with MPO plus 0.3 mU of GO per ml to generate extracellular H_2O_2 (Fig. 1, right-hand panels). Similar results were obtained with human recombinant MPO for isolate LAI- or ELI-infected CEM cells (total of 17 experiments; data not shown).

In the above experiment, 0.1 mM NaI was added; however, MPO was nearly as effective without NaI, i.e., in the presence of Cl^- (data not shown).

While these experiments measured essentially cell-released virus, in other experiments the cell-associated virus (see Materials and Methods) was similarly inactivated by low concentrations of MPO or LPO (data not shown).

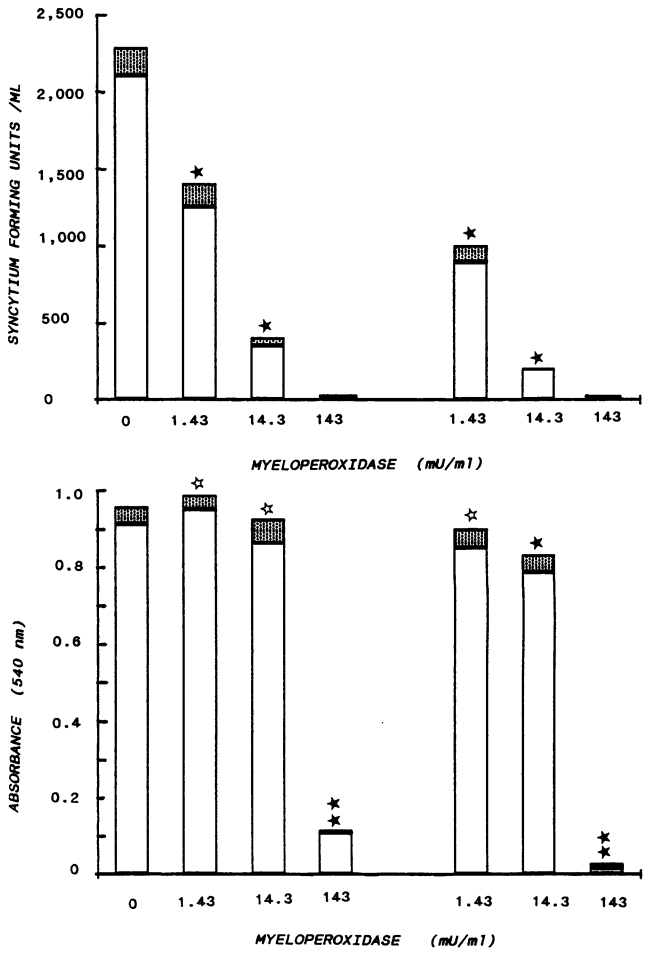

FIG. 1. Virucidal effect of MPO on HIV-1-infected CEM cells. Cultures were initiated with washed CEM cells persistently infected with isolate LAI (10^5 cells per ml). On day 0, 1.43, 14.3, or 143 mU of MPO per ml in sterile saline containing 0.1 mM NaI (left-hand panels) or diluted in sterile saline, 0.1 mM NaI, and 0.3 mU of GO per ml (right-hand panels) was added to duplicate wells. The titer of infectious supernatant virus (top) and the cell viability (bottom) were determined after 4 days of culture. Each bar is the mean and standard deviation (SD) (stippled area over the bar) of duplicate (for syncytium-forming units) or triplicate (for viability) wells. ☆, $P > 0.05$; ★, $P = 0.05$ to 0.005; ★★, $P < 0.001$.

Uninfected CEM cells, unlike the HIV-1-infected cells, were not susceptible to the cytotoxic effects of MPO or LPO at any of the concentrations tested (data not shown). Their viability was unaffected by 143 to 286 mU of MPO per ml with or without 0.1 mM NaI, by 143 to 1,430 mU of LPO per ml with or without NaI, or by 0.3 mU of GO per ml. These concentrations are far in excess of those that are virucidal for HIV-1.

Virucidal properties of MPO on HIV-1-infected human PBMC. Because only the ELI isolate had tropism for the PBMC, as indicated by released infectious virus in day 14 supernatants, subsequent experiments were carried out with this isolate.

MPO was virucidal against ELI-infected human PBMC (Fig. 2). The virucidal effect was selective, since PBMC viability was unaffected or only marginally affected (Fig. 2B) by 1.43 to 71.5

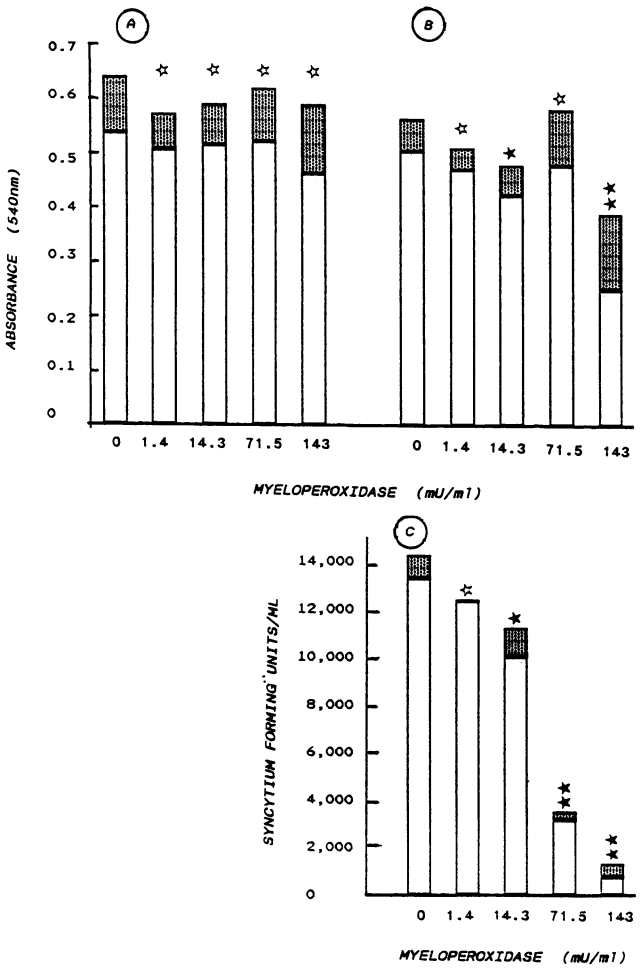

FIG. 2. Virucidal effect of MPO on HIV-1-infected human PBMC. Normal PBMC were stimulated with PHA, infected with isolate ELI, and cultured with or without MPO. (A) Viability of uninfected control PBMC; (B) viability of ELI-infected PBMC; (C) infectious virus titer of ELI-infected PBMC. Each bar is the mean and SD of three experiments. See the legend to Fig. 1 for further explanation.

mU of MPO per ml, which inactivated up to 75% of the ELI isolate (Fig. 2C). However, at 143 mU/ml, MPO was also toxic for the infected PBMC, killing approximately 50% of the cells (Fig. 2B).

MPO was not toxic for uninfected PBMC at any of the concentrations tested (Fig. 2A). In other experiments, the viability of uninfected 3-day-old PHA-stimulated human PBMC was not affected by growing the cells in up to 715 mU of MPO per ml for 4 days (data not shown).

Inhibition of the peroxidase-mediated virucidal effect by catalase in persistently infected CEM cells. We investigated the mechanism responsible for the MPO-mediated virucidal effect, using the enzymatic H_2O_2 scavenger, catalase. Addition of catalase to CEM cultures at the same time as 143 mU of MPO per ml significantly inhibited the virucidal effect of MPO in a concentration-dependent fashion (Fig. 3). MPO inactivated CEM-associated HIV-1 to undetectable levels in the absence of catalase, but MPO plus 10,000 U of catalase per ml raised the viral titer to around 50% of that in untreated control cells. The P24 antigen concentration, a somewhat less sensitive indicator of viral replication, was similarly augmented in the

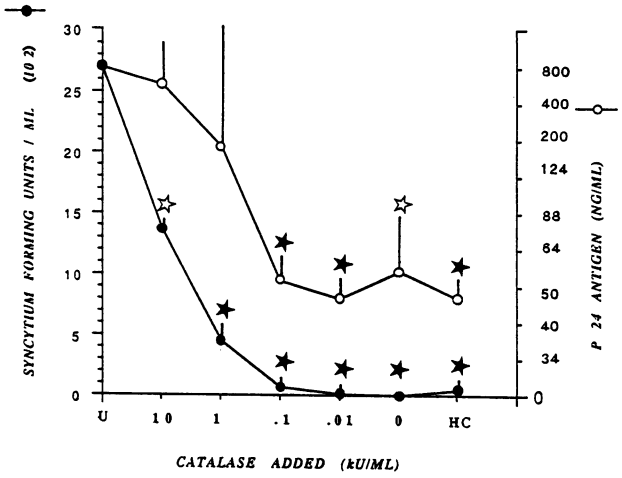

FIG. 3. Inhibition of the MPO-mediated virucidal effect by catalase. MPO (143 mU/ml) and the indicated amounts of catalase were added to cultures of persistently infected CEM cells on day 0. Supernatant infectious virus and P24 antigen were determined on day 4. Each datum point is the mean and SD of duplicate determinations. U, untreated control culture; HC, 5 kU of catalase heat inactivated by boiling 15 min at 100°C; ☆, $P = 0.01$; ★, $P < 0.01$. Similar results were obtained in two other experiments.

presence of catalase (Fig. 3). The viability of the CEM cells in these experiments was also nearly 100% after incubation with MPO plus catalase (data not shown). Heat-inactivated catalase did not have these protective effects.

DISCUSSION

The results of the present study show that cell-associated HIV-1 in CEM lymphoblastoid T cells or PBMC is sensitive to components of the peroxidase-H_2O_2-halide system. In this model system, human MPO, either purified from HL-60 cells or recombinant MPO, is virucidal when added to cultures of HIV-1-infected CEM cells or HIV-1-infected PBMC. The inactivation of HIV appears selective, since the virus is inactivated by low doses of MPO without cell viability being affected. In a previous study, the viability of HIV-1-infected U937 monocytoid cells was similarly unaffected during culture in the presence of recombinant MPO (18). Higher concentrations of MPO (≥143 mU/ml) are toxic to infected cells. However, MPO cytotoxicity is specific in that uninfected CEM cells and PBMC are not affected by virucidal or cytotoxic amounts of MPO and resist high concentrations of the enzyme.

Mammalian peroxidases are found in secretions, such as milk, saliva, and vaginal fluids, in which they play an important role as antimicrobial agents. To investigate whether the amounts of MPO that we found to be virucidal in vitro have any relevance to the amounts found in vivo, we measured the salivary peroxidase activity in seven healthy individuals. We found an average of 0.56 U of peroxidase activity per ml of saliva (range, 0.035 to 1.9 U/ml) (unpublished results). These values are within the range of MPO activity that has a potent in vitro anti-HIV-1 effect. Wide ranges of peroxidase activity have likewise been found in vaginal fluids (13, 16), in which peroxidase could help prevent sexual transmission of HIV.

Since lentiviruses are fully assembled at the cell membrane and are released in free form by budding (8), the virus is probably inactivated by the MPO at the cell membrane proper. In the virucidal system, MPO catalizes the oxidation of Cl^- to

hypochlorous acid, which in turn may react with amino acids to form chloramines. Chloramines decompose spontaneously into NH_3, CO_2, Cl^-, and aldehyde (5, 10, 15). These and other derivatives of the enzymatic reaction, in addition to a possible direct halogenation of cellular proteins, may be responsible for the observed virucidal effect. Extraneous Cl^- was not added in our experiments, but there was probably enough provided by the NaCl in culture medium. Viral constituents may be oxidized at a critical gp120 cysteine that is needed for CD4 binding (19) or at gp120 carbohydrate residues, rendering the protein sensitive to intracellular proteases.

We previously showed that cell-free HIV-1 is sensitive to inactivation by the peroxidase-H_2O_2-halide system (25). An H_2O_2 source was found to be essential to inactivate cell-free HIV (15). This is usually supplied in the reaction mixture by GO, which generates H_2O_2 from glucose. However, H_2O_2 can also be supplied by activated polymorphonuclear leukocytes (5) or by H_2O_2-producing bacteria, such as *Lactobacillus acidophilus* (13). In contrast, we find that the LAI and ELI isolates in infected cells were susceptible to MPO without an extraneous source of H_2O_2. This raised the question of the origin of H_2O_2, since the peroxidase-H_2O_2-halide system cannot function without it. We therefore reasoned that the only source of H_2O_2 must be the infected cells themselves. In accordance with this, catalase, an enzymatic antioxidant that specifically decomposes H_2O_2, showed partial inhibition of the virucidal effect of MPO and MPO cytotoxicity for infected CEM cells.

We have recently confirmed this hypothesis by a direct search for H_2O_2 in infected CEM cells and PBMC, using the scopoletin fluorescence quenching technique (6, 21). An average of 0.060 nmol of H_2O_2 per 10^6 cells per min was released from LAI- or ELI-infected CEM cells, and an average of 0.016 nmol/10^6 cells per min was released from the ELI-infected PBMC. Only traces of H_2O_2 were detectable in the uninfected control cells (unpublished data). In addition to the well-documented release of H_2O_2 from stimulated polymorphonuclear leukocytes (2), other mammalian cell types, such as tumor cells (11, 23) or endothelial cells (22), have been reported to produce hydrogen peroxide. These results therefore suggest that the infected cells produce sufficient peroxide to effectively drive the virucidal peroxidase-H_2O_2-halide system. The results also provide a rational basis for the observed virucidal and cytotoxic properties of MPO in the absence of added H_2O_2.

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