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Virucidal Effects of Glucose Oxidase and Peroxidase or Their Protein Conjugates on Human Immunodeficiency Virus Type 1

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Glucose oxidase and peroxidase (lactoperoxidase or myeloperoxidase) are virucidal to human immunodeficiency virus type 1 (HIV-1) in the presence of sodium iodide, as assessed by the loss of viral replication in a syncytium-forming assay or by the inhibition of cytopathic effects on infected cells. In the presence of low concentrations of sodium iodide, five HIV-1 isolates were equally susceptible to this virucidal system at enzyme concentrations of a few milliunits. The loss of viral replication was linearly related to the time of incubation in the enzyme solutions, with an inactivation rate of 1 log unit every 30 min. These enzymes and this halide were also cytotoxic to chronically infected, but not to uninfected, cultured CEM cells. Protein conjugates were prepared by using the enzymes and murine antibody 105.34, which recognized the V3 loop of HIV-1 LAI isolate surface glycoprotein, or recombinant human CD4. The protein conjugates inactivated free virus at rates similar to those of the free enzymes and were more effective than antibody or recombinant CD4 alone. These in vitro findings demonstrate that the peroxidase-H_2O_2-halide system provides potent virucidal activity against HIV-1.

Myeloperoxidase (MPO) is one of the antimicrobial agents found in large amounts in neutrophils, which are among the primary effector cells in the host defense mechanisms against microbial infections and certain fungal infections. Activation of the neutrophils leads to a respiratory burst and is accompanied by metabolic H_2O_2 formation (2). In the presence of H_2O_2, peroxidase oxidizes the halogen (the halogen is usually Cl^- in normal in vivo circumstances), with the resultant toxicity probably due to direct halogenation of cellular proteins (16). The peroxidase-H_2O_2-halide system has proven to be highly effective against bacteria, fungi, and mycoplasmas (1). This system was also reported to be virucidal against poliovirus and vaccinia virus (3) and more recently against the human immunodeficiency virus type 1 (HIV-1) isolate LAV-1 (5, 17).

Specific immunotoxins (ITs) have been produced by utilizing monoclonal antibodies (MAb) or the viral CD4 receptor and toxins, such as ricin A chain (20, 25) or *Pseudomonas* exotoxin (6), for in vitro targeting of cells expressing HIV surface glycoprotein (gp120). More-recent work was aimed at specifically preventing HIV-1 replication in infected cells by using ITs containing pokeweed antiviral protein (15, 28). We have previously reported the targeting to normal human T lymphocytes (14) and murine myeloma cells (24) of enzyme ITs containing glucose oxidase (GO) and lactoperoxidase (LPO) in the presence of a halide to mediate cytotoxicity. The rationale for using these ITs was based on the observation of Klebanoff (16) that MPO or LPO, together with H_2O_2 and a halide, form a potent bactericidal system. Such ITs were also shown to be effective as antiinfectious agents in eliminating parasites, various bacteria (1), and yeasts such as *Candida tropicalis* (4).

In the current studies, we have utilized GO, LPO, or MPO, and NaI to inactivate several isolates of cell-free and cell-associated HIV-1. We have also tested specific protein conjugates of these enzymes by coupling them to a murine gp120-specific MAb or to human recombinant soluble CD4 (rCD4), and their antiviral efficiency was evaluated with respect to those of the free enzymes and halide, as well as to free MAb or rCD4.

MATERIALS AND METHODS

HIV-1 isolates and cell lines. HIV-1 isolates MN, RF, and ELI were donated by F. Barré-Sinoussi (Pasteur Institute, Paris, France). HIV-1 LAI, formerly BRU (26), was a gift from F. Barré-Sinoussi and was propagated in our laboratory in Villejuif, France. We named it Vil, as we could differentiate it serologically from a single stock of HIV-1/LAI which came from Y. Rivière (Pasteur Institute), which we thus termed HIV-1/LAI (Pas) (unpublished data).

The HT4 LacZ-1 cell line, provided by J.-F. Nicolas (Pasteur Institute), was produced by transfecting $CD4^+$ HeLa cells with the β-galactosidase gene (22) of *Escherichia coli*. These cells were used to titrate HIV-1 isolates LAI and ELI in a syncytium-forming assay (see below). The cells were maintained in Dulbecco modified Eagle medium (Boehringer Mannheim) containing 10% nondecomplemented fetal calf serum (FCS), penicillin, and streptomycin and then grown at 37°C in a humidified 5% CO_2 atmosphere. The HUT 78 T-cell line, used to estimate the cytopathicity of the MN and RF viral isolates, was obtained from F. Barré-Sinoussi (Institut Pasteur). HIV-1 LAI (Vil) and ELI were

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propagated by chronically infecting CEM T cells at the low multiplicity of infection of 5,000 cpm of reverse transcriptase activity per 10^6 cells. The HUT 78 and CEM cell lines were maintained in RPMI 1640 culture medium containing 10% heat-inactivated FCS, penicillin, streptomycin, 2 mM glutamine, and 1% nonessential amino acids.

Antibodies, enzymes, and protein conjugates. Murine MAb 105.34 (immunoglobulin G1) was specific to the RIQRGP GRAFVT sequence, which represents residues 313 to 324 of the hypervariable segment of the V3 loop of the gp120 protein of HIV-1 LAI, and has a Kd for gp120 of 8.8×10^{-10} M (7). This MAb neutralized HIV-1 LAI (Vil) but not LAI (Pas) (unpublished data). The antibody was a gift of F. Traincard (Institut Pasteur).

Human rCD4 (M_r, 48,000) was a soluble construct containing domains 1 to 4 of CD4. It was a gift from A. F. Williams (University of Oxford, Oxford, England). Human MPO, purified from HL60 cells, a human promyelocytic cell line (4), was a gift of T. Bringman (Ideon Corporation, Redwood City, Calif.).

Protein conjugates were prepared with MAb 105.34 and rCD4, using heterobifunctional cross-linking agents by methods described in detail elsewhere (14, 24). In brief, after derivatizating MAb 105.34 or rCD4 with *S*-acetylmercaptosuccinic anhydride (Sigma) and the enzymes with 4-succinimidyloxycarbonyl-α-methyl-α-(2-pyridyldithio)toluene, a gift from P. E. Thorpe (Imperial Cancer Research Fund, London, England), the conjugates were purified on a column of Ultrogen 34 to separate the reactants (14, 24). GO (M_r, 160,000) from the fungus *Aspergillus niger* (EC 1.1.3.4) was obtained from Biozyme (Gwent, England) and had a specific activity of 180 U/mg of protein. LPO (M_r, 78,000) from bovine milk (EC 1.11.1.8) was from Biozyme or Sigma and had a specific activity of approximately 25 U/mg of protein. The enzymatic activities of GO, LPO, and MPO were estimated by spectrophotometric assays, as previously described (14, 24) and expressed as units of enzyme activity as defined elsewhere (10, 27).

Virus inactivation assay. Inactivation of virus infectivity by the protein conjugates or by free MAb, rCD4, or the free enzymes was carried out essentially as described by Nara et al. (19). In the standard procedure, 0.2- to 2-ml samples of culture supernatants containing suspensions of either LAI (Vil), LAI (Pas), ELI, MN, or RF (3,000 to 20,000 syncytium-forming units [SFU] per ml; 250,000 to 2,300,000 cpm of reverse transcriptase per ml) were supplemented with 1.5 mU of free GO or GO protein conjugate, 1.2 mU of free LPO or LPO protein conjugate, and NaI (final concentration of 0.1 mM) in Falcon 2058 tubes and incubated at 37°C in a humidified atmosphere in a CO_2 incubator. The enzymes were mixed or were added singly. At various times after the addition of enzyme, the residual levels of infectivity of the LAI and ELI isolates were determined in the β-galactosidase syncytium-forming assay on HT4 LacZ-1 indicator cells, as described in detail previously (22), except that 7 μg, instead of 10 μg, of DEAE-dextran was added to each well.

In neutralization assays with free MAb 105.34 or rCD4, the results were expressed as 50% neutralization doses after the curve of residual virus infectivity versus serial dilution of the proteins (10 μg to 1 ng) was plotted.

The infectivity of HIV-1 isolates MN and RF was tested on HUT 78 indicator cells, as follows. Samples (50 μl) of treated or untreated control virus suspensions were added to duplicate wells of 24-well tissue culture plates. The cultures were fed every 4 days with fresh RPMI 1640 culture medium, and when a microscopically visible cytopathic effect was

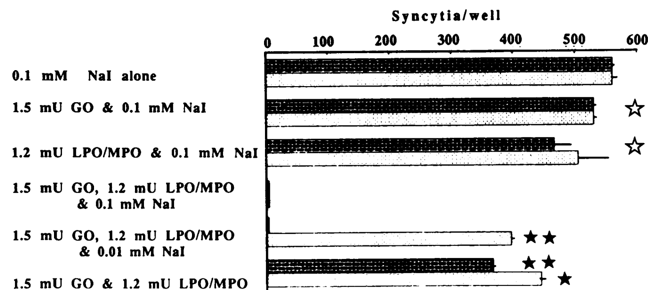

FIG. 1. Inactivation of HIV-1 ELI infectivity by the peroxidase-H_2O_2-halide system. Aliquots of 400 μl of virus suspensions in RPMI 1640 culture medium were treated with the enzymes and NaI indicated for 45 min at 37°C, and residual virus infectivity was estimated in the β-galactosidase syncytium-forming assay (see Materials and Methods). Each bar shows the mean and standard deviation for two wells seeded with 75 μl of virus suspension. Similar results were obtained in four additional experiments. Symbols: bars with black bricks, suspensions with LPO; bars with light stippling, suspensions with MPO; ★★ and ★, significantly different ($P < 0.001$ and $P = 0.01$, respectively) from control (0.1 mM NaI alone); ☆, not significantly different ($P = 0.07$ to 0.32) from control.

seen, at 16 days for MN and 21 days for RF, the cells were collected and washed once, and their viability was determined in a tetrazolium dye-based colorimetric assay, as described previously (24).

ELISA analysis of protein conjugates. The binding of protein conjugates, 105.34-GO, 105.34-LPO, and rCD4-GO or rCD4-LPO, to gp160 was determined by an enzyme-linked immunosorbent assay (ELISA). This assay was done with Immulon 2 plates (Dynatech Laboratories, Chantilly, Va.) coated with gp160 donated by M. Kaczorek (Transgène and Pasteur Mérieux). The enzymatic activities of the bound GO or LPO protein conjugates were then determined by incubating the plates in the presence of the appropriate chromogenic substrate.

RESULTS

Virucidal properties of the peroxidase-H_2O_2-halide system. As shown in Fig. 1, suspensions of HIV-1 ELI supplemented with as little as 1.5 mU of GO plus 1.2 mU of LPO or MPO in 0.4 ml of culture medium in the presence of 0.01 to 0.1 mM of NaI sufficed to inactivate most of the viral infectivity. NaI was less effective when MPO was present. Moreover, a small reduction in the numbers of syncytia (20% for MPO and 35% for LPO) was seen when NaI was omitted from the incubation mixture (bottommost pair of bars in Fig. 1). This reduction was observed in most, but not all, experiments and was significantly different from the control values (topmost pair of bars) when it occurred. It probably represents the contribution of the Cl^- present in the medium to the toxicity of the system.

Virus inactivation was little affected when the FCS concentration in the virus incubation mixture was raised, from 10 to 77% (data not shown). In this range of enzyme and NaI concentrations, the treated viral suspensions were not toxic when added to the HT4 LacZ-1 indicator cells because of the 13.3-fold dilution effect (see the legend to Fig. 1). As a general rule, cellular cytotoxicity was critically dependent on the GO concentration and only amounts greater than 1.5 mU/ml of culture medium proved to be toxic to these cells or to phytohemagglutinin-stimulated normal T lymphocytes (data not shown).

TABLE 1. HIV-1 inactivation by free MAb 105.34, rCD4, and the peroxidase-H_2O_2-halide system[a]

Time after treatment	Infectivity[b] of HIV-1 after treatment			
	None	MAb 105.34	rCD4	GO-LPO-NaI system
Immediately after	642/100	1/100, 1/100	0/100, 0/100	0/100, 0/100
Day 7	38/150	0/50, 0/150	0/50, 1/150	0/50, 0/150
Day 11	561/50	5/50, 18/150	2/50, 5/150	0/50, 0/150
Day 14	336/30	435/30	423/30	0/30

[a] Aliquots of 400 μl of HIV-1 LAI (Vil) suspensions in RPMI 1640 culture medium (5,000 SFU/ml) were treated with 210 μg of MAb 105.34 (486 50% neutralization doses), with 12.6 μg of rCD4 (170 50% neutralization doses), or with 3 mU of GO, 2.4 mU of LPO, and 0.1 mM NaI (final concentration), or were left untreated. After 45 min at 37°C, residual infectivity was assayed as described in Materials and Methods section.

[b] The numbers are SFU per the one or two wells scored on the day indicated/microliters of virus suspension inoculated in each well on day 0.

The virucidal efficiency of the peroxidase-H_2O_2-halide system was also assessed by inoculating aliquots into CEM cells in order to amplify the replication of any remaining intact virus particles. At intervals of 7, 11, and 14 days after inoculation, aliquots of supernatant were collected and assayed in the usual syncytium-forming assay. As shown in Table 1, at no time after treatment of HIV-1 LAI (Vil) with the GO-LPO-NaI system was residual viral infectivity detected. On the other hand, control treatments with either antibody or rCD4 allowed residual virus to persist; virus was barely detectable immediately after treatment but clearly present at later times. Similar results were obtained with the LAI (Pas) isolate.

Inactivation of HIV-1 isolates LAI (Vil), LAI (Pas), and ELI was also studied as a function of the length of time of incubation in the presence of GO, LPO, and NaI. Incubation of 20,000 SFU/ml in the peroxidase-H_2O_2-halide system inactivated virus in a manner that was linearly related to time of incubation at 37°C (Fig. 2). From the curve of SFU versus time in Fig. 2, we were able to calculate a rate of inactivation of 1 log unit every 30 to 35 min after an initial lag period of approximately 60 min during which time no decrease in the number of syncytia occurred.

Inactivation of HIV-1 MN and RF by the peroxidase-H_2O_2-halide system. Since the MN and RF isolates formed syncytia poorly, we assessed their susceptibility to the peroxidase-H_2O_2-halide system by an assay measuring their cytopathic effect on HUT 78 cells. Treatment of the virus suspensions with GO, LPO, and NaI strongly reduced their ability to replicate in and kill HUT 78 cells within the 16- or 21-day culture period (Fig. 3). This time period was necessary, because it took this long for a cytopathic effect to become visible in cultures infected with untreated control virus (bars labeled HIV-1 only in Fig. 3). Thus, the growth of HUT 78 cells was affected little or not at all by inoculation with virus suspensions that had been treated with the peroxidase-H_2O_2-halide system (bars labeled HIV-1, 1.5 mU GO & 1.2 mU LPO), and the growth of the cells was similar to the growth of uninfected control cells (Fig. 3). This result indicated that most of the virus had been inactivated by the enzymatic treatment. As expected, the treatments 1.5 mU of GO alone and 1.2 mU of LPO alone with 0.1 mM NaI were not virucidal.

Inactivation of the RF isolate was also confirmed in a radioimmunoassay specifically detecting gp160 of HIV-1. We found viral antigen in day 21 culture supernatants of HUT 78 cells infected with untreated control RF suspensions but not in supernatants of GO-, LPO-, and NaI-treated suspensions (data not shown).

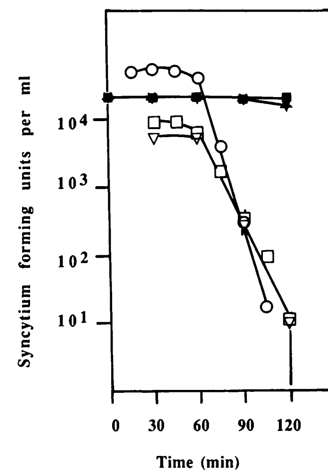

FIG. 2. Time-dependent inactivation of HIV-1 infectivity by the peroxidase-H_2O_2-halide system. Samples (1 to 2 ml) of suspensions of HIV-1 isolates LAI (Vil) or ELI in RPMI 1640 culture medium were treated with the enzymes and NaI (see Materials and Methods). At the times indicated, aliquots were withdrawn and residual virus infectivity was estimated by the β-galactosidase syncytium-forming assay. Control virus suspensions were incubated in medium and 0.1 mM NaI alone. Each point shows the mean and standard deviation for two wells seeded with 10 or 75 μl of virus suspension. Similar results were obtained in two additional experiments. Symbols: open circles and open triangles, two independent experiments with HIV-1 LAI (Vil); open squares, HIV-1 ELI; solid symbols, untreated control suspensions of HIV-1 LAI (Vil) or ELI.

Selective cytotoxicity of the peroxidase-H_2O_2-halide system on CEM cells chronically infected with HIV-1. As shown in Fig. 4a, most of the chronically infected CEM cells were killed after 4 days of culture in medium supplemented with 0.3 mU of GO, 10 mU of LPO, and 0.1 mM of NaI. Unexpectedly, however, we found that the cells were as susceptible to treatment with 10 mU of LPO and NaI, i.e., no GO added (Fig. 4, panel a). This cytotoxicity was selective, since uninfected control CEM cells were unaf-

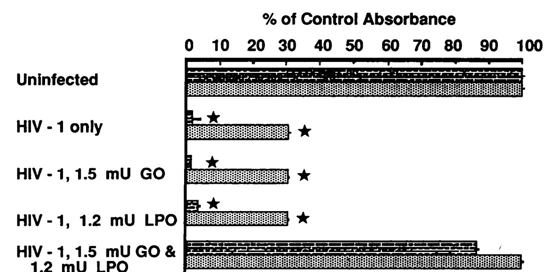

FIG. 3. Inactivation of HIV-1 isolates MN and RF by the peroxidase-H_2O_2-halide system. Viral suspensions in RPMI 1640 culture medium were treated with GO or LPO in the presence of 0.1 mM NaI, as indicated, and the suspensions were used to inoculate single wells containing HUT 78 cells. After 16 to 21 days of growth, the viability of the cells was assessed in a tetrazolium-based colorimetric assay. Each bar shows the mean and standard deviation for three wells. Symbols: dark bars, isolate MN; stippled bars, isolate RF; ★, significantly different ($P < 0.001$) from control (uninfected) (other conditions were not significantly different).

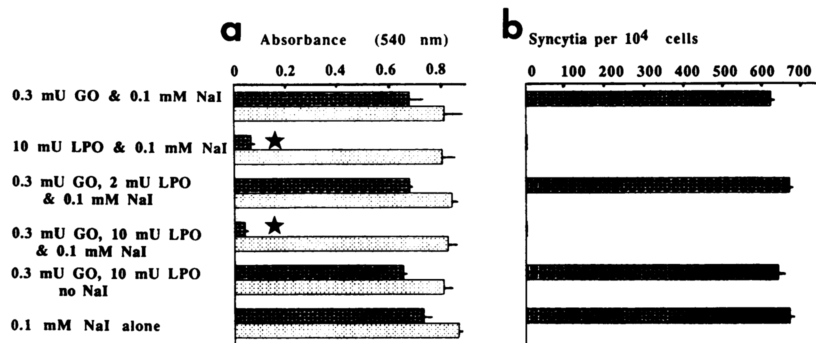

FIG. 4. Cytotoxicity of the peroxidase-H_2O_2-halide system against CEM cells chronically infected with HIV LAI (Vil). (a) Infected CEM cells (bars with black bricks) or uninfected control CEM cells (bars with light stippling) received the indicated supplements per ml of culture medium. After 4 days of growth, the cells were collected and washed, and the aliquots were assayed for viability in a tetrazolium-based colorimetric assay. Each bar shows the mean and standard deviation for three wells. ⋆, significantly different ($P < 0.001$) from control (0.1 mM NaI alone) (other conditions were not significantly different). (b) Aliquots of 10^4 washed cells or cellular debris in an equivalent volume were assayed for the presence of infectious virus in the β-galactosidase syncytium-forming assay. Each bar shows the mean and standard deviation for two wells. Similar results were obtained in seven additional experiments.

fected by these treatments (Fig. 4, panel a, lightly stippled bars).

Figure 4b shows that the cell-associated virus of these cells was inactivated by treatment with GO, LPO, and NaI or with LPO and NaI. Thus, the conditions that were cytotoxic to the cells were also virucidal.

Characterization of purified MAb 105.34 and rCD4 protein conjugates. The purified conjugate fractions that eluted immediately after the void volume of the column gel were pooled separately into two fractions designated early and late fractions and were analyzed by ELISA and by sodium dodecyl sulfate–7.5% polyacrylamide gel electrophoresis under nonreducing conditions to avoid rupturing the disulfide links introduced between the targeting proteins and the enzymes. These experiments indicated a strong and specific binding of the protein conjugates to the gp160 protein coated on Immulon 2 plates (data not shown). We concluded that the protein conjugates specifically recognized gp120 while retaining their enzymatic activity.

We calculated coupling ratios (enzyme molecules to antibody or rCD4 molecules) of approximately 1.5 to 2:1 for the early eluting fraction and 1:1 for the late eluting fraction. These ratios were based on the conjugate's apparent molecular weight, as estimated from gel chromatography elution profiles and from the migration of proteins on sodium dodecyl sulfate-polyacrylamide gels and a calculation of the ratio of enzyme activity to A_{280} for each conjugate, knowing the extinction coefficients of the proteins.

Virucidal effects of protein conjugates. When the protein conjugates had been adjusted to contain the same activities of GO and LPO as in the free enzyme-halide system, the rates of inactivation of HIV-1 LAI (Vil) decreased linearly with time, with a reduction in titer of approximately 1 log unit every 30 min. Inactivation kinetics were similar whether the virus was targeted with the protein conjugates or with the free enzymes (Fig. 5). However, treating the virus with protein conjugates resulted in a shorter lag period of approximately 30 min before virus inactivation could be detected compared with those of the free enzymes.

In addition, the MAb 105.34 and rCD4 protein conjugates were more efficient than free MAb or rCD4 in inactivating HIV-1 LAI (Vil). For example, while the rCD4 protein conjugate lowered the initial titer of 8,900 SFU/ml to 2 SFU/ml in 2 h of incubation, free rCD4 reduced the titer to only 3,940/ml by 2 h (Fig. 5B). Another experiment performed on isolate LAI (Pas) yielded similar results.

DISCUSSION

In this study, we have shown that GO- and LPO-containing HIV-1-specific protein conjugates or the free components of the peroxidase-H_2O_2-halide system are potent virucidal agents against HIV-1. This system requires the combined presence of an oxidase, a peroxidase, and halide

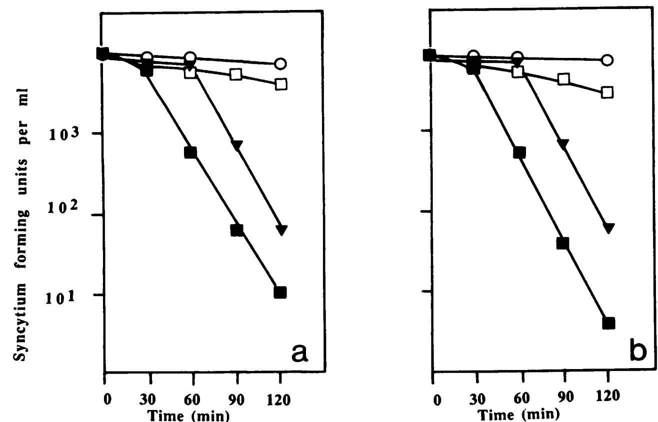

FIG. 5. Comparison of the virucidal power of protein conjugates, free MAb 105.34 or rCD4, and the free components of the peroxidase-H_2O_2-halide system. Suspensions of HIV-1 LAI (Vil) in culture medium were incubated with 105.34-GO and 105.34-LPO (a) or with rCD4-GO and rCD4-LPO (b) and 0.1 mM NaI at the same enzyme activities as in Fig. 1, or with free GO and LPO at the same final concentration in the presence of 0.1 mM NaI at 37°C. Free MAb 105.34 was adjusted to a concentration of 308 ng/ml, and the concentration of free rCD4 was 192 ng/ml. Both these reagents were at the same final concentration as in the protein conjugates. Untreated control virus suspensions were incubated in culture medium and 0.1 mM NaI. Residual virus infectivity was estimated in the β-galactosidase syncytium-forming assay. Each point indicates the SFU/milliliter of a single well. Symbols: solid squares, 105.34-GO plus 105.34-LPO (panel a) and rCD4-GO plus rCD4-LPO (panel b); solid triangles, free GO plus LPO; open squares, free MAb 105.34 (panel a) and free rCD4 (panel b); open circles, untreated control.

to generate antiviral toxicity and has been previously used in enzyme ITs to kill tumor cells and various microorganisms (1, 4, 14, 16, 24). In the in vitro assays, presented here, GO and glucose produced H_2O_2, which then oxidized the added iodide salt in the presence of LPO or MPO.

A recent study showed that Cl^- was highly effective in inactivating HIV-1 in vitro (17), whereas we showed here that I^- was more effective. In this study, the inactivation obtained in the absence of added iodide, which is presumably due to excess Cl^- present in the medium, was only part of the inactivation caused by iodide against HIV-1 LAI or ELI. We suggest that differences in incubation conditions, for example the use of polymorphonuclear leukocytes to generate H_2O_2 (17), could account for the difference. It should be mentioned, however, in support of our findings that another study showed that I^-, but not Cl^-, with free MPO or LPO was effective in inactivating vaccinia virus and poliovirus (3).

This toxicity-generating system differs basically from previously described ricin A chain- or pokeweed-containing anti-HIV-1 ITs (15, 28) in that it can directly attack free virus. Thus, free virus present in secretions, such as semen (8), or circulating in blood might now be considered a potential target of this virucidal system. Although the results presented here were obtained in vitro, notice that raising the concentration of FCS in the RPMI medium to 77% did not affect the virucidal power of this system.

The finding that the peroxidase-H_2O_2-halide system exhibited selective cytotoxicity against the chronically infected CEM cells, in addition to inactivating the cell-associated virus, appears to be novel. We had failed in our initial attempts to obtain specific cytotoxicity against these cells by targeting with the protein conjugates. Thus, the conjugates were no more cytotoxic than the free enzymes and halide were (data not shown). This failure was presumably due to a low surface representation of the gp120 viral antigenic material, as we also failed to demonstrate surface staining using free MAb 105.34 or rCD4.

The fact that the chronically infected CEM cells were susceptible to GO and LPO in the presence of NaI suggests that the cells were somehow stressed by the infection and perhaps more fragile. Furthermore, the susceptibility of these cells to LPO and NaI alone, in the absence of H_2O_2 generation by GO, suggests that the cells produce by themselves sufficient peroxide to initiate the cytotoxicity of the system. Some supportive evidence that HIV-infected cells suffer from a state of oxygen stress comes from reports that HIV-infected individuals have abnormally low levels of intracellular reduced glutathione (11), and some T-cell subsets with high levels of glutathione were not present in these individuals (23). Reduced glutathione is known to protect cells from oxidative stress by scavenging H_2O_2 (13).

The effectiveness of this virucidal system was also reported in another study which showed as much as a 10,000-fold reduction of the infectivity titer of poliovirus (3). We found a 1-log-unit reduction of viral infectivity for every 30 min (approximately) of incubation (Fig. 2 and 5). In contrast, other experiments (Fig. 1) showed more than 2 log units of virus inactivation in 45 min, without a lag period. The different conditions employed may account for this discrepancy. Tubes with small incubation volumes (0.2 to 0.4 ml) were probably better oxygenated and provided more optimal conditions for GO activity, thus inactivating virus more rapidly. On the other hand, the repeated removal (every 15 or 30 min) of tubes and of the HT4 Lac-Z1 cells from the CO_2 incubator (Fig. 2 and 5), may have decreased the efficacy of the virucidal system, which is influenced by pH and temperature, and may have been detrimental to optimal virus adherence to and penetration into the HT4 Lac-Z1 cells, resulting in the observed lag before virus inactivation.

A comparison of the peroxidase-H_2O_2-halide system with free MAb or rCD4 showed that large doses of the free MAb or rCD4 (see footnote *a* of Table 1) were not as effective as the GO-LPO-NaI combination in inactivating HIV-1 LAI. Furthermore, in the experiment in which we measured virus inactivation as a function of the length of time of incubation (Fig. 5), the protein conjugates or the free enzymes and halide were more effective than equal amounts of free MAb or rCD4.

However, one concern regarding the in vivo application of this approach is the toxicity of GO (9). Its 50% lethal dose in mice is 6 to 12.8 μg/g of body weight (18; also unpublished data). LPO and MPO are much less toxic (50% lethal dose of >32 μg/g). The high-mannose level of GO giving preferential uptake in the liver is thought to cause the in vivo toxicity (9). However, the fact that chronically infected CEM cells are susceptible to MPO and halide in the absence of GO (Fig. 4) suggests that a treatment protocol without GO might be feasible.

The finding that human MPO could effectively replace bovine LPO may be important. MPO is a natural human enzyme which is nontoxic in the presence of physiological amounts of Cl^- and which should not to be immunogenic. The possible virucidal role of peroxidase in human extracellular fluids was demonstrated in a recent study which showed HIV-1 inactivation in saliva supplemented with GO and glucose (21). Moreover, the ability of mucosal secretions to inactivate HIV-1 is presently of major interest (12), as it was reported that semen from HIV-1-infected humans also contains HIV (8). Our in vitro findings demonstrate that the peroxidase-H_2O_2-halide system has potential virucidal activity against HIV-1.

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REFERENCES

1. **Arnold, R. R.** 1985. Antibody targeting of the antimicrobial and antitumor activity of the peroxidases, p. 229–241. *In* K. P. Pruitt and J. O. Tenuovo (ed.), The lactoperoxidase system, vol. 27. Marcel Dekker, Inc., New York.
2. **Babior, B. M., J. T. Curnutte, and N. Okamura.** 1988. The respiratory burst oxidase of the human neutrophil, p. 43–48. *In* B. Halliwell (ed.), Oxygen radicals and tissue injury. The Upjohn Co., Bethesda, Md.
3. **Belding, M. E., S. J. Klebanoff, and C. G. Ray.** 1970. Peroxidase-mediated virucidal systems. Science **167:**195–196.
4. **Casentini-Borocz, D., and T. Bringman.** 1990. Enzyme immunoconjugates utilizing glucose oxidase and myeloperoxidase are cytotoxic to *Candida tropicalis*. Antimicrob. Agents Chemother. **34:**875–880.
5. **Chase, M. J., and S. J. Klebanoff.** 1992. Viricidal effect of stimulated human mononuclear phagocytes on human immunodeficiency virus type 1. Proc. Natl. Acad. Sci. USA **89:**5582–5585.
6. **Chaudhary, V. K., T. Mizukami, T. R. Fuerst, D. J. FitzGerald, B. Moss, I. Pastan, and E. A. Berger.** 1988. Selective killing of HIV-infected cells by recombinant human CD4-*Pseudomonas* exotoxin hybrid protein. Nature (London) **335:**369–372.
7. **Chouchane, L., T. Bringman, S. Barbier, F. Traincard, and A. D. Strosberg.** 1990. Targeted killing of yeast expressing a HIV-1 peptide by antibody-conjugated glucose oxidase and

horseradish peroxidase. Immunol. Lett. **25**:359–366.
8. **Coombs, R. W., J. N. Krieger, A. C. Collier, S. O. Ross, K. Chalupka, V. J. Murphy, D. K. Cummings, and L. Corey.** 1992. Plasma viremia and recovery of HIV from semen: implications for transmission and therapy, p. 145–149. *In* F. Melica (ed.), AIDS and human reproduction. Karger, Basel.
9. **Demignot, S., and D. Domurado.** 1987. Effect of prosthetic sugar groups on the pharmacokinetics of glucose oxidase. Drug Design Deliv. **1**:333–348.
10. **Dionysius, D. A., P. A. Grieve, and A. C. Voss.** 1992. Studies on the lactoperoxidase system: reaction kinetics and antibacterial activity using two methods for hydrogen peroxide generation. J. Appl. Bacteriol. **72**:146–153.
11. **Eck, H.-E., H. Gmunder, M. Hartmann, D. Petzoldt, V. Daniel, and W. Droge.** 1989. Low concentrations of acid-soluble thiol (cysteine) in the blood plasma of HIV-1-infected patients. Biol. Chem. Hoppe-Seyler **370**:101–108.
12. **Fultz, P. N.** 1986. Components of saliva inactivate human immunodeficiency virus. Lancet **ii**:1215.
13. **Halliwell, B., and J. M. C. Gutteridge.** 1989. Free radicals in biology and medicine, 2nd ed. p. 102. Clarendon Press, Oxford.
14. **Ito, H., J. Morizet, L. Coulombel, and M. Stanislawski.** 1990. T cell depletion of human bone marrow using an oxidase-peroxidase enzyme immunotoxin. Bone Marrow Transplant. **6**:395–398.
15. **Kim, Y.-W., M. S. C. Fung, N.-C. Sun, C. R. Y. Sun, N. T. Chang, and T. W. Chang.** 1990. Immunoconjugates that neutralize HIV virions kill T cells infected with diverse strains of HIV-1. J. Immunol. **144**:1257–1262.
16. **Klebanoff, S. J.** 1967. Iodination of bacteria: a bactericidal mechanism. J. Exp. Med. **126**:1063–1078.
17. **Klebanoff, S. J., and R. W. Coombs.** 1992. Viricidal effect of polymorphonuclear leucocytes on human immunodeficiency virus-1. Role of the myeloperoxidase system. J. Clin. Invest. **89**:2014–2017.
18. **Nakane, P. L., and T. Watanabe.** 1984. Distribution of oncodevelopmental markers in neoplastic tissues. J. Histochem. Cytochem. **32**:894–898.
19. **Nara, P. L., W. C. Hatch, N. M. Dunlop, W. G. Robey, L. O. Arthur, M. A. Gonda, and P. L. Fischinger.** 1987. Simple, rapid, quantitative syncytium-forming microassay for the detection of human immunodeficiency virus neutralizing antibody. AIDS Res. Hum. Retroviruses **3**:283–302.
20. **Pincus, S. H., K. Wehrly, and B. Chesbro.** 1989. Treatment of HIV tissue culture infection with monoclonal antibody-ricin A chain conjugates. J. Immunol. **142**:3070–3075.
21. **Pourtois, M., C. Binet, N. Van Tieghem, P. R. Courtois, A. Vandenabbele, and L. Thirty.** 1991. Saliva can contribute in quick inhibition of HIV infectivity. AIDS **5**:589–600.
22. **Rocancourt, D., C. Bonnerot, H. Jouin, M. Emerman, and J.-F. Nicolas.** 1990. Activation of a β-galactosidase recombinant provirus: application to titration of human immunodeficiency virus (HIV) and HIV-infected cells. J. Virol. **64**:2660–2668.
23. **Roederer, M., F. J. T. Staal, H. Osada, L. A. Herzenberg, and L. A. Herzenberg.** 1991. CD4 and CD8 T cells with high intracellular glutathione are selectively lost as the HIV infection progresses. Int. Immunol. **3**:933–937.
24. **Stanislawski, M., V. Rousseau, M. Goavec, and H. Ito.** 1989. Immunotoxins containing glucose oxidase and lactoperoxidase with tumoricidal properties: in vitro killing effectiveness in a mouse plasmacytoma cell model. Cancer Res. **49**:5497–5504.
25. **Till, M. A., V. Ghetie, T. Gregory, E. J. Patzner, J. P. Porter, J. W. Uhr, D. J. Capon, and E. S. Vitetta.** 1988. HIV-infected cells are killed by rCD4-ricin A chain. Science **242**:1166–1168.
26. **Wain-Hobson, S., J.-P. Vartanian, M. Henry, N. Chenciner, R. Cheynier, S. Delassus, L. P. Martins, M. Sala, M.-T. Nugeyre, D. Guetard, D. Klatzmann, J.-C. Gluckman, W. Rozenbaum, F. Barre-Sinoussi, and L. Montagnier.** 1991. LAV revisited: origins of the early isolates from Institut Pasteur. Science **252**:961–965.
27. **Worthington Biochemical Corp.** 1982. Worthington enzyme manual. Worthington Biochemical Corp., Freehold, N.J.
28. **Zarling, J. M., P. A. Moran, O. Haffar, J. Sias, D. D. Richman, C. A. Spina, D. E. Myers, V. Kuebelbeck, J. A. Ledbetter, and F. M. Uckun.** 1990. Inhibition of HIV replication by pokeweed antiviral protein targeted to $CD4^+$ cells by monoclonal antibodies. Nature (London) **347**:92–95.