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Inactivation of Human Immunodeficiency Virus Type 1 by the Amine Oxidase-Peroxidase System

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Human immunodeficiency virus type 1 (HIV-1) is rapidly inactivated by exposure to a naturally occurring antimicrobial system consisting of peroxidase, H_2O_2, and a halide. Among the potential sources of H_2O_2 is the amine oxidase system in which mono-, di-, and polyamines are oxidatively deaminated with the formation of H_2O_2. The polyamine spermine is present at exceptionally high concentrations in semen. We report here that spermine, spermidine, and, to a lesser degree, the synthetic polyamine 15-deoxyspergualin are viricidal to HIV-1 when combined with amine oxidase and myeloperoxidase. Antiviral activity required each component of the spermine-amine oxidase-peroxidase system and was inhibited by azide (a peroxidase inhibitor) and by catalase but not by superoxide dismutase. Heat treatment of catalase largely abolished its inhibitory effect. These findings implicate H_2O_2 formed by the amine oxidase system in the antiviral effect and raise the possibility that the polyamine-amine oxidase-peroxidase system influences the survival of HIV-1 in semen and in the vaginal canal.

H_2O_2 at a relatively high concentration (10^{-2} M) is viricidal to human immunodeficiency virus type 1 (HIV-1), and when the concentration of H_2O_2 is lowered to a level at which it is ineffective alone, further addition of peroxidase and a halide restores antiviral activity. The peroxidases shown to be effective in this regard are myeloperoxidase (MPO) (7, 23, 30, 54), eosinophil peroxidase (23), and lactoperoxidase (37, 38). MPO can be added as the purified enzyme from neutrophils (23) or HL-60 cells (7, 54) or as a recombinant precursor protein (7, 30). It also can be released from stimulated neutrophils (24) or monocytes (6). The lactoperoxidase can be obtained from milk (54) or saliva (37, 38). H_2O_2 can be added as reagent H_2O_2 (23) or be generated by glucose and glucose oxidase (30, 37, 38, 54), lactobacilli (23), or stimulated phagocytes (6, 24). The halide can be chloride, bromide, iodide, or the pseudohalide thiocyanate, with differences in halide specificity among the peroxidases. The halide is oxidized by peroxidase and H_2O_2 to form a strong oxidant, such as the corresponding hypohalous acid or halogen, with toxic properties (22). Although most studies were performed with cell-free HIV-1, toxicity to T-cell-associated virus also has been reported (7).

Amine oxidases are a heterogeneous group of enzymes which can catalyze the oxidative deamination of mono-, di-, and polyamines with formation of the corresponding aldehyde, H_2O_2, and with oxidation of a primary amine, ammonia, as follows: $RCH_2NH_2 + O_2 + H_2O \rightarrow RCHO + H_2O_2 + NH_3$. Oxidation of a secondary amine releases a more complex nitrogen-containing compound: $R_1CH_2NHCH_2R_2 + O_2 + H_2O \rightarrow R_1CHO + H_2O_2 + H_2NCH_2R_2$. Amine oxidases differ in substrate specificity and in inhibitor profile and can be either flavin adenine dinucleotide- or Cu-containing enzymes (31).

The polyamine spermine is present in semen at high concentrations with levels greater than 10^{-3} M having been reported (11, 17, 21, 29, 45, 52). Spermidine is also present in seminal plasma, although at considerably lower concentrations, and trace amounts of putrescine also have been detected (29). Although the spermine of semen is predominantly in the seminal plasma, appreciable amounts of this polyamine also are present in spermatozoa (39).

We report here on the viricidal effect on HIV-1 of spermine, spermidine, and, to a lesser degree, the synthetic polyamine 15-deoxyspergualin when combined with amine oxidase and MPO.

MATERIALS AND METHODS

Special reagents. Amine oxidase (monoamine oxidase from bovine serum; catalog no. M4636), spermine tetrahydrochloride, spermidine tetrahydrochloride, putrescine dihydrochloride, cadaverine dihydrochloride, scopoletin, and horseradish peroxidase (type VI; 250 to 330 U/mg) were obtained from Sigma Chemical Co., St. Louis, Mo. Bovine liver catalase (57,622 U/mg) was from Worthington Biochemical Corp., Freehold, N.J., and superoxide dismutase (SOD; 5,000 U/mg) from bovine erythrocytes was from Boehringer Mannheim Biochemicals, Indianapolis, Ind. 15-Deoxyspergualin trihydrochloride was kindly provided by the Bristol Myers-Squibb Co. Pharmaceutical Research Institute, Seattle, Wash. MPO was isolated from human neutrophils (41) and assayed by guaiacol oxidation (26). The catalase was dialyzed against water overnight and heated at 100°C for 20 min where indicated.

H_2O_2 production. Production of H_2O_2 by the amine oxidase system was determined by measurement of scopoletin oxidation (42). The components indicated in the legend to Fig. 1 were incubated at 37°C for the periods indicated in a temperature-controlled cuvette with constant stirring, and the change in fluorescence intensity (excitation wavelength, 350 nm; emission wavelength, 460 nm) was determined with a fluorescence spectrophotometer (model LS-5; Perkin-Elmer, Norwalk, Conn.).

HIV-1 viricidal activity. HIV-1_{LAI} (kindly provided by Genetic Systems, Seattle, Wash.) was propagated in CEM cells (CCL 119; American Type Culture Collection, Rockville, Md.) in RPMI 1640 (Gibco Laboratories, Grand Island, N.Y.) containing 10% fetal calf serum (Gibco), 50 U of penicillin per ml, 50 μg of streptomycin per ml, 0.01% DEAE dextran (M_r, 500,000 Sigma Chemical Co.), and 0.01 M *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) buffer (pH 6.8) (CEM growth medium). A stock viral preparation of 10^6 50% tissue culture-infective doses per ml in CEM growth medium was frozen at −70°C and, just prior to the experiment, diluted 100-fold in 0.1 M sodium sulfate before a further 10-fold dilution in the reaction mixture to give a final concentration of 1,000 50% tissue culture-infective doses per ml. The reaction mixture, containing the components indicated in the figure legends and the table footnote (in addition to all of the components present in CEM growth medium diluted 1,000-fold) in a final volume of 0.5 ml, was incubated in sterile screw-cap microtubes (4.3 by 10.8 mm, 1.5-ml capacity; model 75.692.005; Sarstedt, Inc., Princeton, N.J.) with sealing O rings for 30 min at 37°C in a CO_2 incubator. A 10-μl volume was transferred to 48-well plates (model 3548; Costar, Data-Packaging Corp., Cambridge, Mass.) containing 2×10^5 CEM cells in 1 ml of CEM growth medium. After incubation in a CO_2 incubator at 37°C for 7 days, a 200-μl aliquot was removed for measurement of HIV-1 p24 antigen by solid-phase sandwich-

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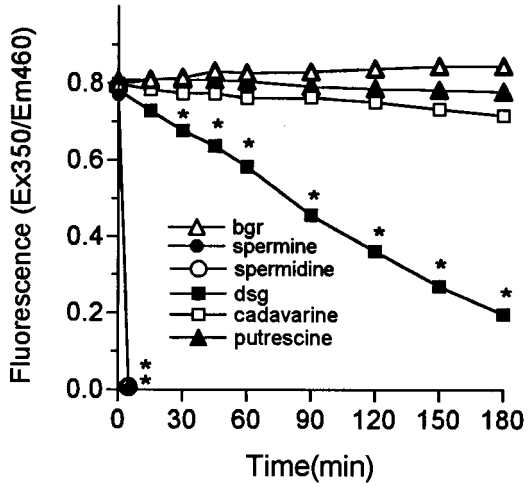

FIG. 1. H_2O_2 production by the amine oxidase system. The reaction mixture contained 0.02 M sodium phosphate buffer (pH 7.0), 4×10^{-6} M scopoletin, 9.6 μg of horseradish peroxidase per ml either alone (background [bgr]) or supplemented with 23 μg (2.3 mU) of amine oxidase per ml, and 10^{-4} M spermine, spermidine, 15-deoxyspergualin (dsg), cadaverine, or putrescine in a final volume of 2.5 ml. The results shown are means of three to seven experiments. An asterisk indicates a significant difference from the background ($P < 0.05$). Ex350, excitation wavelength of 350 nm; Em460, emission wavelength of 460 nm.

type enzyme-linked immunosorbent assay (Abbott Laboratories, Chicago, Ill.). We prepared controls in which a comparable dilution of HIV-1 was added to wells lacking CEM cells and incubated for 7 days. The values, which indicated the amounts of p24 present in the added virus, were always less than 100 ng/ml (mean ± the standard error of the mean, 61.3 ± 4.7; $n = 13$). A positive p24 antigen cutoff of 10 pg/ml was used.

Statistical analyses. H_2O_2 determinations are expressed as means, and the significance of the differences was determined by Student's two-tailed t test for independent means. HIV-1 p24 antigen levels are expressed as medians, and the two-tailed Mann-Whitney U rank-sum test was used to analyze differences for significance ($P > 0.05$ was deemed not significant).

RESULTS

H_2O_2 production. Figure 1 demonstrates the formation of H_2O_2 by the amine oxidase system as measured by the horseradish peroxidase-catalyzed oxidation of scopoletin. When spermine or spermidine was employed as the substrate, H_2O_2 production was rapid and the scopoletin was completely oxidized in 5 min. No H_2O_2 production was detected with cadaverine or putrescine as the substrate under our experimental conditions, whereas intermediate levels of H_2O_2 were formed when 15-deoxyspergualin was employed.

HIV-1 viricidal activity. Table 1 demonstrates the viricidal effect on HIV-1 of the amine oxidase system when combined with MPO and chloride as measured by the inability of the virus to replicate in CEM cells. No significant viricidal activity was observed with either cadaverine or putrescine as the amine oxidase substrate; with 15-deoxyspergualin, there was a partial decrease in p24 antigen production, whereas when spermine or spermidine was employed, the ability of HIV-1 to replicate in CEM cells was essentially lost. The HIV-1 viricidal activity of the spermine-amine oxidase-MPO-Cl^- system was prevented by omission of spermine, amine oxidase, or MPO from the reaction mixture; however, when chloride was omitted, only a small (but significant) loss of activity was observed. The viricidal activity of the complete system was inhibited by the peroxidase inhibitor azide and by catalase, but not by SOD. Catalase inhibition was largely lost on heat treatment. Azide, catalase, or SOD alone, i.e., in the absence of the amine oxidase system, had no effect on viral replication in CEM cells under these conditions. A viricidal effect of the spermine-amine oxidase-MPO system was observed at spermine concentrations as low as 3×10^{-7} M (Fig. 2).

TABLE 1. Viricidal effect of the amine oxidase-peroxidase system on HIV-1[a]

Additions	Median HIV-1 p24 antigen level	P^1	P^2
None	166,000 (27)		
Cadaverine + amine oxidase + MPO + Cl^-	129,900 (6)	NS	
Putrescine + amine oxidase + MPO + Cl^-	81,368 (6)	NS	
15-Deoxyspergualine + amine oxidase + MPO + Cl^-	30,600 (6)	<0.01	
Spermidine + amine oxidase + MPO + Cl^-	24 (6)	<0.001	
Spermidine + amine oxidase + MPO + Cl^-	11 (24)	<0.001	
Spermine + amine oxidase + MPO + Cl^- with:			
Spermine omitted	256,000 (9)	NS	<0.001
Amine oxidase omitted	158,500 (9)	NS	<0.001
MPO omitted	244,000 (9)	NS	<0.001
Cl^- omitted	24 (12)	<0.001	<0.05
Azide added	40,700 (9)	<0.05	<0.001
Catalase added	315,500 (6)	NS	<0.001
Heated catalase added	39 (6)	<0.001	<0.002
SOD added	9 (6)	<0.001	<0.05

[a] The reaction mixture contained 4×10^{-2} M sodium phosphate buffer (pH 7.0), 1,000 50% tissue culture-infective doses of HIV-1_{LAI} per ml, and, where indicated, 10^{-5} M cadaverine, putrescine, 15-deoxyspergualin, spermidine, or spermine; 23 μg (2.3 mU) of amine oxidase per ml; 226 mU of MPO per ml; 10^{-2} M NaCl; 10^{-4} M sodium azide; 85 μg of catalase per ml; and 10 μg of SOD per ml in a final volume of 0.5 ml. The catalase was heated at 100°C for 20 min where indicated. The results are expressed (in picograms per milliliter) as the median of the number of values shown in parentheses. P^1 is the P value for the difference from no addition (none), and P^2 is the P value for the difference from the complete spermine-amine oxidase-MPO-Cl^- system. NS, not significant.

DISCUSSION

Oxidation of spermine, spermidine, and, to a lesser degree, 15-deoxyspergualin by amine oxidase results in formation of H_2O_2 which, when combined with MPO and chloride, is viricidal to HIV-1. Our studies were limited to a laboratory-adapted strain of HIV-1 (HIV-1_{LAI}), and it is recognized that primary isolates of HIV-1 can differ in their responses to immunosuppressive agents. Although an absolute requirement for the polyamine, amine oxidase, and MPO was demonstrated under our experimental conditions, chloride deletion had only a small effect. The latter finding may indicate the involvement of a product other than hypochlorous acid in the antiviral activity or the presence of adequate chloride in the other reagents. In this regard, small amounts of chloride were added with the polyamines which were hydrochloride salts.

An inhibitory effect of catalase which is largely prevented by heat inactivation (Table 1) implicates the H_2O_2 formed by the spermine-amine oxidase system in the HIV-1 viricidal effect. In some other systems, toxicity by amine oxidase has been found to be catalase insensitive, and in most of these instances, toxicity has been ascribed to reactive aminoaldehydes or another degradation product of the oxidized amine (12, 13, 28, 43). In some systems, both H_2O_2 and aminoaldehydes have been implicated (4), and ammonia also has been proposed as a toxic product of the amine oxidase system (15). The polyamine con-

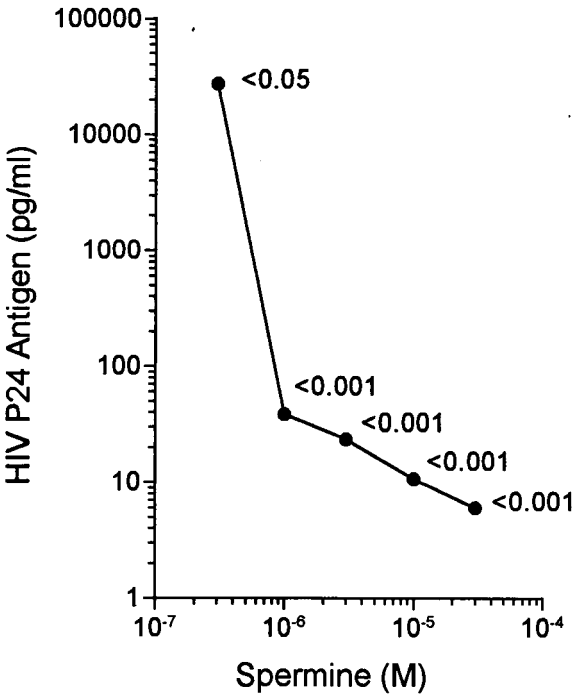

FIG. 2. Effect of catalase. The reaction mixture was as described for the spermine-amine oxidase-MPO-Cl system in the footnote to Table 1, except that the spermine concentration was varied as indicated. The results shown are medians of 6 to 24 values. The *P* values for the difference from no additions (none in Table 1; 166,000 pg/ml; $n = 27$) are shown.

centration generally employed in our study was 10^{-5} M, which, if completely oxidized by amine oxidase, would produce equimolar concentrations of H_2O_2 and ammonia. H_2O_2 alone (i.e., in the absence of peroxidase) at this concentration was not viricidal, although a higher concentration (10^{-2} M) did have an antiviral effect. Similarly, ammonium chloride was not viricidal at concentrations ranging from 10^{-6} to 10^{-3} M (data not shown). Our findings indicate that when the concentration of H_2O_2 formed by the amine oxidase system is inadequate for viricidal activity, further addition of a peroxidase may induce H_2O_2-dependent damage.

The high concentration of polyamines in seminal plasma has stimulated interest in their possible role there. Seminal plasma has been reported to have immunosuppressive and cytotoxic properties (20) which, in some systems, were dependent on the bovine serum present as a component of the tissue culture medium (2, 3, 40, 51). Bovine serum contains an amine oxidase at high concentrations. It is a copper-containing enzyme with a high affinity for spermine and spermidine (36). Because of its reaction with monoamines such as benzylamine, it has been called monoamine oxidase and its high activity with the polyamines spermine and spermidine has led to the term polyamine oxidase or spermine oxidase. The immunosuppressive properties of seminal plasma when combined with bovine serum have been attributed to a product of the interaction of seminal plasma polyamines and the bovine serum amine oxidase (1–3, 5). 15-Deoxyspergualin also has immunosuppressive properties (46, 47). In one study, the antiproliferative activity of 15-deoxyspergualin when combined with bovine serum was inhibited by an amine oxidase inhibitor, whereas the activity in the presence of human serum, which has low amine oxidase levels, was unaffected by the amine oxidase inhibitor (27).

Amine oxidases with affinity for the polyamines spermine and spermidine are widely distributed (32, 44). The amine oxidase generally employed in studies of the immunosuppressive properties of seminal plasma, and that employed here, is from bovine serum. This enzyme is found at high levels in the sera of ruminants but is absent or is present in small amounts in nonruminants. With more sensitive methods of detection, small amounts of amine oxidase have been found in male and nonpregnant female sera and this activity is increased considerably in the serum of pregnant women (16, 19, 35) and in retroplacental serum obtained at delivery (18, 34). A polyamine oxidase also has been detected in human monocytes (14, 15, 33). Human seminal plasma contains an amine oxidase (9, 10, 17, 21, 55, 56) which, unlike the amine oxidase of bovine serum, has highest affinity for diamines such as putrescine (thus the term diamine oxidase), although it also can react with the polyamines spermine and spermidine. A peroxidase also has been detected in human seminal plasma (10) and is present in the vaginal fluid of most women (25, 48, 49) in amounts sufficient to produce an in vitro microbicidal effect (25). The latter may be of uterine origin or be released from neutrophils, monocytes, or eosinophils in the region.

A role for the amine oxidase system in the transmission of HIV-1 has been proposed (53) on the basis of very high levels of spermine in seminal plasma and the immunosuppressive properties of seminal plasma when combined with an amine oxidase. Further, polyamine levels are increased in cells infected with cytomegalovirus (50), an infection often associated with HIV-1, and the polyamine level in lymphocytes is increased in patients with HIV-1 infection (8). The findings reported here raise the possibility that components of the polyamine-amine oxidase-peroxidase system provided by either seminal plasma or vaginal fluid can influence the transmission of HIV-1 by affecting its survival following intercourse.

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