

Viricidal Effect of Polymorphonuclear Leukocytes on Human Immunodeficiency Virus-1

Role of the Myeloperoxidase System

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Abstract

Myeloperoxidase (MPO), H_2O_2 , and chloride form an antimicrobial system in neutrophilic polymorphonuclear leukocytes (PMN) effective against a variety of microorganisms. Normal human PMN, when stimulated with phorbol myristate acetate or opsonized zymosan, are viricidal to HIV-1. The viricidal effect was lost when chloride was replaced by sulfate and was inhibited by the peroxidase inhibitor azide and by catalase, but not by heated catalase or superoxide dismutase, implicating H_2O_2 . Stimulated PMN from patients with chronic granulomatous disease (CGD) were not viricidal to HIV unless H_2O_2 or glucose oxidase (which generates H_2O_2) was added, and the viricidal activity of H_2O_2 -supplemented CGD PMN was inhibited by azide, implicating endogenous MPO. Stimulated PMN from patients with hereditary MPO deficiency had decreased viricidal activity unless MPO was added, and the viricidal activity of MPO-supplemented, MPO-deficient PMN was inhibited by catalase, implicating endogenous H_2O_2 . The data suggest that when PMN are stimulated, MPO released by degranulation reacts with H_2O_2 formed by the respiratory burst to oxidize chloride to a product (presumably hypochlorous acid) that is toxic to HIV-1. Our findings raise the possibility that this viricidal effect of stimulated PMN may influence the host defense against HIV-1. (*J. Clin. Invest.* 1992; 89:2014–2017.)
Key words: neutrophil • chronic granulomatous disease • hereditary myeloperoxidase deficiency • AIDS • hypochlorous acid • HIV

Introduction

We have previously reported that the cell-free myeloperoxidase (MPO)¹- H_2O_2 -halide system is viricidal to HIV-1 and that H_2O_2 -generating *Lactobacillus acidophilus* can provide the H_2O_2 required for this system (1). Since lactobacilli and peroxi-

dase are present in the vagina of most normal women (2–4), the possibility was raised that heterosexual transmission of HIV-1 may be influenced by the presence and level of the peroxidase-mediated antimicrobial system in the vagina.

MPO is present in large amounts in cytoplasmic granules of neutrophilic polymorphonuclear leukocytes (PMN) and is released into either the phagosome or the extracellular fluid when PMN are stimulated by a variety of soluble or particulate stimuli (5, 6). Stimulation of PMN also results in a respiratory burst in which oxygen is reduced first to the superoxide anion (O_2^-) and then to H_2O_2 . MPO, H_2O_2 , and chloride form an antimicrobial system in PMN through the formation of powerful oxidants, namely, hypochlorous acid and related chloramines (5, 6). We report here that intact-stimulated PMN are viricidal to HIV-1, and provide evidence for the involvement of the MPO- H_2O_2 -chloride system in this toxicity.

Methods

Reagents. The lymphadenopathy associated virus-1 (LAV-1) strain of HIV-1, kindly provided by Genetic Systems, Seattle, WA, was propagated in CEM cells (Genetic Systems) in RPMI-1640 (Gibco Laboratories, Grand Island, NY) containing 10% fetal calf serum (Gibco Laboratories), 50 U/ml penicillin, 50 μ g/ml streptomycin, 0.01% DEAE Dextran (500,000 M_r , Sigma Chem. Co., St. Louis, MO) and 0.01 M HEPES buffer pH 6.8 (CEM growth medium). A stock viral preparation of 10^6 tissue culture infective dose (TCID)₅₀/ml in CEM growth medium was frozen at -70°C and on the day of the experiment was diluted 100-fold in 0.1 M sodium sulfate before a further 10-fold dilution in the reaction mixture (final concentration 1,000 TCID₅₀/ml). In addition to the components listed in the table legends, the reaction mixture contained all the components present in CEM growth medium diluted 1,000-fold. Catalase (CTR, bovine liver, 84,150 U/mg) was obtained from Worthington Biochemical Corp. (Freehold, NJ), phorbol myristate acetate (PMA), Histopaque-1077, superoxide dismutase (bovine erythrocyte, 3,150 U/mg) and glucose oxidase (*Aspergillus niger* type V) from Sigma Chem. Co. The catalase was dialyzed against water overnight before use and was heated at 100°C for 20 min where indicated. The PMA was dissolved in dimethylsulfoxide and the concentration of the latter in the reaction mixture did not exceed 0.01%. MPO was purified from human leukocytes (7) and assayed by guaiacol oxidation (8). Zymosan (ICN Pharmaceuticals, Inc., Cleveland, OH) was suspended in water by homogenization, boiled for 20 min, washed twice, and suspended in water at 10 mg/ml. The zymosan was opsonized by incubation with an equal volume of pooled normal serum for 20 min at 37°C with shaking, and the opsonized zymosan was washed twice and suspended in water at 10 mg/ml.

Isolation of PMN. Venous blood was collected from normal volunteers, two patients with chronic granulomatous disease (CGD) and one patient with hereditary MPO deficiency, just before the experiment, using 0.2% EDTA as anticoagulant. The PMN were isolated by dextran sedimentation and centrifugation in Histopaque-1077, and the erythrocytes removed by hypotonic lysis (9). The cells that always contained

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1. *Abbreviations used in this paper:* CGD, chronic granulomatous disease; MPO, myeloperoxidase; TCID, tissue culture infective dose.

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greater than 97% PMN with an average purity of 98–99% were suspended in 0.9% sodium chloride at 5×10^7 PMN/ml. The final PMN preparation contained 4.9% eosinophils (average of five determinations) with the remainder neutrophils.

Measurement of viricidal activity. The components of the reaction mixture (see legends) in a final volume of 0.5 ml were incubated in sterile screwcap microtubes with sealing O rings (4.3×10.8 mm, 1.5 ml capacity, model 75.692.005; Sarstedt, Inc., Princeton, NJ) for 30 min at 37°C in a CO₂ incubator. The tubes were centrifuged to sediment the cells and 10 µl of the supernatant fluid was added to 48-well plates (model 3548; Costar, Data Packaging Corp., Cambridge, MA) containing 2×10^5 CEM cells in 1 ml of CEM growth medium. After incubation in a CO₂ incubator at 37°C for 6 d, a 200-µl aliquot was removed for measurement of HIV-1 P24 antigen by solid phase sandwich-type enzyme-linked immunosorbent assay (Abbott Laboratories, Chicago, IL). In general, culture supernatant levels of HIV-p24 antigen were either zero (< 10 pg/ml), indicating no replication, or very high, indicating unrestricted replication, with the level reached being related to the number of CEM cells (2×10^5), the volume of the reaction mixture added to the CEM cells (10 µl), and the growth period (6 d). In initial experiments, dilution and remeasurement of p24 antigen was not performed when the maximum optical density of > 2.000 (> 604 pg/ml) was obtained in the initial assay. These data are included in the tables under “No. with HIV Growth”/“No. of Samples,” with an optical density of > 2.000 considered to indicate growth. In subsequent experiments, when an optical density of > 2.000 was observed, the supernatant fraction was diluted and remeasured. The data are expressed as the median p24 antigen level (picogram/milliliter) above background for those samples in which dilutions were performed when necessary. A positive HIV p24 antigen cutoff of 10 pg/ml was used. Median values were employed because of the general distribution of the values into two populations, representing no growth and extensive replication. Where indicated, statistical analysis was performed using the Mann-Whitney U test. Not significant (NS) $P > 0.05$.

Results and Discussion

Normal PMN, when combined with the soluble stimulus PMA or the particulate stimulus opsonized zymosan, were viricidal to HIV-1, as measured by an inability to replicate in CEM cells (Table I). No viricidal effect was evident when either PMA or opsonized zymosan was added alone, whereas a decrease ($P = 0.002$) but not loss of p24 antigen production was observed on incubation with PMN alone. The viricidal effect of PMA- or opsonized zymosan-stimulated PMN was lost when chloride was replaced by sulfate in the incubation medium. The viricidal effect was inhibited by azide, a potent inhibitor of MPO, and by catalase, but not by heated catalase, indicating a requirement for H₂O₂. Superoxide dismutase was not inhibitory, indicating that O₂⁻ was not required for the viricidal effect. These properties are compatible with the release of MPO by degranulation and the formation of H₂O₂ by the respiratory burst and their interaction, presumably in the extracellular fluid, with chloride to form an agent toxic to HIV-1.

Additional support for the involvement of the MPO-H₂O₂-chloride system in the viricidal effect of intact PMN on HIV-1 came from patients with a hereditary defect in PMN function. CGD is a rare genetic disorder in which PMN lack a respiratory burst due to the absence of a functional nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (10–12). As a result, the toxic products of the respiratory burst, including H₂O₂ and oxidants dependent on H₂O₂ for their production, are not formed. The MPO content of CGD PMN, however, is normal. When normal PMN were replaced by PMN from patients with CGD, toxicity to HIV-1 with either PMA or opsonized zymo-

Table I. Viricidal Effect of Stimulated Normal PMN on HIV-1

Supplements	HIV-1 P24 antigen	
	Median pg/ml of (n) samples	No. with HIV-1 growth/ No. of samples
None	493,480 (27)*	27/27
PMN	59,910 (14)*	19/20
PMA	300,250 (9)	15/15
PMN + PMA	<10 (32) [§]	4/36
Chloride deleted	390,390 (6)	6/6
Azide added	5,050 (9) [‡]	16/18
Catalase added	12,350 (9) [§]	14/18
Heated catalase added	<10 (12)	1/12
SOD added	<10 (12)	0/12
Ops zym	594,290 (5)	12/12
PMN + ops zym	<10 (29)	10/36
Chloride deleted	997,700 (5)	6/6
Azide added	209,780 (6)	18/18
Catalase added	215,160 (6)	18/18
Heated catalase added	<10 (8)	6/12
SOD added	<10 (10)	2/12

The reaction mixture contained 5×10^{-3} M sodium phosphate buffer pH 7.4, 0.128 M NaCl, 1.2×10^{-2} M KCl, 10^{-3} M CaCl₂, 2×10^{-3} M MgCl₂, 2×10^{-3} M glucose, 1,000 TCID₅₀/ml HIV-1 and, where indicated, 10⁶/ml PMN, 100 ng/ml PMA, 1 mg/ml opsonized zymosan, 10^{-4} M sodium azide, 58 µg/ml catalase, and 10 µg/ml superoxide dismutase. In tubes in which chloride was deleted, isosmolar amounts of sulfate were added. The results are expressed as the median P24 antigen level in pg/ml and the ratio of the number of samples in which viral replication occurred over the total number of samples. * $P = 0.002$; ‡ $P = 0.01$; § $P = 0.03$.

san as the stimulus was lost (Table II). This loss of viricidal activity was reversed by the addition of H₂O₂ or glucose oxidase, but not by the addition of MPO. Glucose oxidase generates H₂O₂ in the course of the oxidation of its substrate glucose, and the involvement of the H₂O₂ so formed in the viricidal activity was indicated by the inhibitory effect of catalase but not heated catalase. At the concentration employed, H₂O₂ alone or combined with unstimulated CGD PMN (i.e., without PMA or opsonized zymosan) was not viricidal to HIV-1 (data not shown). The HIV-1 viricidal activity of H₂O₂ (or glucose oxidase)-supplemented CGD PMN stimulated with PMA or opsonized zymosan was inhibited by azide, implicating endogenous MPO released by stimulus-induced degranulation.

The PMN of patients with hereditary MPO deficiency lack MPO, but, in sharp contrast to CGD PMN, respond to stimulation with a respiratory burst which is greater than normal (6, 13). The viricidal effect on HIV-1 was greatly decreased when stimulated normal PMN were replaced by PMA- or opsonized zymosan-stimulated MPO-deficient PMN (Table III). In contrast to the findings with CGD PMN, the loss of viricidal activity was unaffected by the addition of H₂O₂, but was prevented by the addition of MPO. MPO alone or combined with unstimulated MPO-deficient PMN were without effect on HIV-1 (data not shown). The HIV-1 viricidal activity of MPO-supplemented, MPO-deficient PMN stimulated with PMA or opsonized zymosan was inhibited by catalase but not heated catalase, implicating H₂O₂ generated by the stimulus-induced respiratory burst.

Table II. Viricidal Effect of Stimulated CGD PMN on HIV-1

Supplements	HIV-1 P24 antigen	
	Median pg/ml of (n) samples	No. with HIV-1 growth No. of samples
None	493,480 (27)	27/27
CGD PMN + PMA	366,700 (6)*	9/9
MPO added	44,260 (9)*	9/12
H ₂ O ₂ added	<10 (8)	2/9
H ₂ O ₂ and azide added	505,330 (6)	9/9
GO added	<10 (9)	1/9
GO + catalase added	279,710 (6)	6/6
GO + heated catalase added	<10 (6)	0/6
GO + azide added	502,830 (6)	6/6
CGD PMN + ops zym	309,270 (6)*§	9/9
MPO added	25,680 (9)‡	9/12
H ₂ O ₂ added	<10 (7)	2/8
H ₂ O ₂ + azide added	524,170 (6)	9/9
GO added	<10 (9)	0/9
GO + catalase added	51,380 (6)§	6/6
GO + heated catalase added	<10 (6)	0/6
GO + azide added	470,000 (6)	6/6

The reaction mixture was as described in Table I, except that CGD PMN were employed and 0.23 U/ml MPO, 10⁻⁴ M H₂O₂, 10⁻⁴ M sodium azide, 0.03 U/ml glucose oxidase (GO), and 58 µg/ml catalase were added where indicated. * NS; ‡ NS; § NS.

In the MPO-H₂O₂-chloride system, H₂O₂ combines with MPO to form a complex which oxidizes chloride to form the powerful oxidant hypochlorous acid (HOCl) or its dissociated form, the hypochlorite anion (OCl⁻) (5). The pKa of the dissociation is 7.53 so that both forms are present at physiologic pH. When the pH falls, HOCl predominates and HOCl can react with excess chloride to form chlorine (Cl₂). HOCl can also react with nitrogen-containing compounds to form mono- and dichloramines which retain oxidizing activity. Some of the chloramines are relatively long-lived, which provides a mechanism for the prolongation of the oxidizing activity of the MPO system and for the penetration of oxidants into fluids rich in proteins and other scavengers of the more reactive oxidants. HOCl/OCl⁻ (Clorox® bleach) is a well-recognized disinfectant effective against a variety of organisms, including HIV-1 (14).

Holmberg et al. (15) have classified the biological factors that affect sexual transmission of HIV-1 into: (a) the infectiousness of the HIV-1-infected donor; (b) the infectivity and virulence of the virus; and (c) host susceptibility to infection. Although the importance of these factors has been inferred from epidemiologic studies, the specific host defense mechanisms against HIV-1 are poorly understood. Our findings raise the possibility that among the factors that influence host defense against HIV-1 are the products of stimulated phagocytes. Destruction of microorganisms by PMN can occur intracellularly, i.e., within a phagosome, or extracellularly. Neutrophils lack CD4 and therefore the uptake of HIV-1 by this receptor would not be expected. The uptake of antibody-associated HIV-1 by Fc receptor-mediated endocytosis is possible. However, our studies were performed in the absence of specific antibody. It is probable that under our experimental conditions, stimulation of PMN resulted in the release of MPO and H₂O₂ into the

Table III. Viricidal Effect of Stimulated MPO-Deficient PMN on HIV-1

Supplements	HIV-1 P24 antigen	
	Median pg/ml of (n) samples	No. with HIV-1 growth No. of samples
None	493,480 (27)*§	27/27
MPO-def PMN + PMA	98,110 (6)*§	9/9
H ₂ O ₂ added	59,270 (6)‡	9/9
MPO added	<10 (9)	0/9
MPO + catalase added	137,910 (6)*	9/9
MPO + heated catalase added	<10 (9)	0/9
MPO-def PMN + ops zym	336,670 (6)	9/9
H ₂ O ₂ added	470,730 (6)	9/9
MPO added	<10 (9)	0/9
MPO + catalase added	503,860 (6)	9/9
MPO + heated catalase added	<10 (9)	1/9

The reaction mixture was described in Table I, except that MPO-deficient PMN were employed and 10⁻⁴ M H₂O₂, 0.23 U/ml MPO and 58 µg/ml catalase were added where indicated. * P = 0.05; ‡ NS; § P = 0.01.

extracellular fluid where they reacted with chloride to form agents toxic to HIV-1. Stimulation of PMN at mucosal surfaces or at sites of inflammation due to exposure to a variety of chemotactic factors, cytokines, or opsonized microorganisms would be expected, and toxicity to adjacent cell-free HIV-1 may occur under these circumstances. The precise microenvironment in which toxicity to HIV-1 by PMN may occur is not known; factors such as pH, protein concentration, and low molecular weight inhibitors may influence the viricidal effect in vivo. Cell-free HIV-1 has been demonstrated in plasma (16) and seminal fluid (17) and, thus, may be susceptible to attack by extracellular products of stimulated PMN in these and other locations. Provirus or intact cell-associated HIV-1 virions would be expected to be protected from these extracellular oxidants. It is not clear whether disruption of the cell-free pool of HIV-1 would influence the transmission of HIV-1 or the progression of disease. It is of interest in this regard that vaginal transmission of cell-free simian immunodeficiency virus (SIV) (18, 19) or HIV-1 (20) has been demonstrated in nonhuman primates, and that cell-free HIV-1 in plasma is a sensitive marker of the clinical stage of infection (16).

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References

1. Klebanoff, S. J., and R. W. Coombs. 1991. Viricidal effect of *Lactobacillus acidophilus* on human immunodeficiency virus type 1: possible role in heterosexual transmission. *J. Exp. Med.* 174:289-292.
2. Tsibris, J. C. M., S. D. Virgin, F. S. Khan-Dawood, P. W. Langenberg, J. L. Thomason, and W. N. Spellacy. 1986. Cervicovaginal peroxidases: markers of the fertile period. *Obstet. Gynecol.* 67:316-320.
3. Eschenbach, D. A., P. R. Davick, B. L. Williams, S. J. Klebanoff, K. Young-Smith, C. M. Critchlow, and K. K. Holmes. 1989. Prevalence of hydrogen

- peroxide-producing *Lactobacillus* species in normal women and women with bacterial vaginosis. *J. Clin. Microbiol.* 27:251–256.
4. Klebanoff, S. J., S. L. Hillier, D. A. Eschenbach, and A. M. Waltersdorff. 1991. Control of the microbial flora of the vagina by H₂O₂-generating lactobacilli. *J. Infect. Dis.* 164:94–100.
 5. Klebanoff, S. J. 1988. Phagocytic cells: products of oxygen metabolism. In *Inflammation: Basic Principles and Clinical Correlates*. J. I. Gallin, I. M. Goldstein, and R. Snyderman, editors. Raven Press, New York. 391–444.
 6. Klebanoff, S. J. 1991. Myeloperoxidase: occurrence and biological function. In *Peroxidases in Chemistry and Biology*. Volume I. J. Everse, K. E. Everse, and M. B. Grisham, editors. CRC Press, Boca Raton: 1–35.
 7. Rakita, R. M., B. R. Michel, and H. Rosen. 1990. Differential inactivation of *Escherichia coli* membrane dehydrogenases by a myeloperoxidase-mediated antimicrobial system. *Biochemistry*. 29:1075–1080.
 8. Klebanoff, S. J., A. M. Waltersdorff, and H. Rosen. 1984. Antimicrobial activity of myeloperoxidase. *Methods Enzymol.* 105:399–403.
 9. Clark, R. A., and S. J. Klebanoff. 1979. Role of the myeloperoxidase-H₂O₂-halide system in concanavalin A-induced tumor cell killing by human neutrophils. *J. Immunol.* 122:2605–2610.
 10. Malech, H. L., and J. I. Gallin. 1987. Current concepts: immunology. Neutrophils in human diseases. *N. Engl. J. Med.* 317:687–694.
 11. Lehrer, R. I., T. Ganz, M. E. Selsted, B. M. Babior, and J. T. Curnutte. 1988. Neutrophils and host defense. *Ann. Intern. Med.* 109:127–142.
 12. Clark, R. A. 1990. The human neutrophil respiratory burst oxidase. *J. Infect. Dis.* 161:1140–1147.
 13. Nauseef, W. M. 1988. Myeloperoxidase deficiency. *Hematol. Oncol. Clin. North Am.* 2:135–158.
 14. Martin, L. S., J. S. McDougal, and S. L. Loskoski. 1985. Disinfection and inactivation of the human T lymphotropic virus type III/lymphadenopathy-associated virus. *J. Infect. Dis.* 152:400–403.
 15. Holmberg, S. D., C. R. Horsburgh, Jr., J. W. Ward, and H. W. Jaffe. 1989. Biologic factors in the sexual transmission of human immunodeficiency virus. *J. Infect. Dis.* 160:116–125.
 16. Coombs, R. W., A. C. Collier, J.-P. Allain, B. Nikora, M. Leuther, G. F. Gjerret, and L. Corey. 1989. Plasma viremia in human immunodeficiency virus infection. *N. Engl. J. Med.* 321:1626–1631.
 17. Borzy, M. S., R. S. Connell, and A. A. Kiessling. 1988. Detection of human immunodeficiency virus in cell-free seminal fluid. *J. Acquired Immune Defic. Syndr.* 1:419–424.
 18. Miller, C. J., N. J. Alexander, S. Sutjipto, A. A. Lackner, A. Gettie, A. G. Hendrickx, L. J. Lowenstine, M. Jennings, and P. A. Marx. 1989. Genital mucosal transmission of simian immunodeficiency virus: animal model for heterosexual transmission of human immunodeficiency virus. *J. Virol.* 63:4277–4284.
 19. Miller, C. J., N. J. Alexander, S. Sutjipto, S. M. Joye, A. G. Hendrickx, M. Jennings, and P. A. Marx. 1990. Effect of virus dose and nonoxynol-9 on the genital transmission of SIV in rhesus macaques. *J. Med. Primatol.* 19:401–409.
 20. Fultz, P. N., H. M. McClure, H. Daugharty, A. Brodie, C. R. McGrath, B. Swenson, and D. P. Francis. 1986. Vaginal transmission of human immunodeficiency virus (HIV) to a chimpanzee. *J. Infect. Dis.* 154:896–900.