**Brief Definitive Report**

**Viricidal Effect of Lactobacillus acidophilus on Human Immunodeficiency Virus Type 1: Possible Role in Heterosexual Transmission**

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**Summary**

Peroxidase, H$_2$O$_2$, and a halide form a powerful antimicrobial system in phagocytes and tissue fluids, and certain microorganisms can serve as the source of H$_2$O$_2$ for this system. H$_2$O$_2$-generating *Lactobacillus acidophilus* (LB') is present in the vagina of most normal women and peroxidase has been detected in vaginal fluid. LB' at high concentration is viricidal to HIV-1, and, at levels where LB' is ineffective alone, the addition of peroxidase (myeloperoxidase, eosinophil peroxidase) and a halide (chloride, iodide, bromide, thiocyanate) restore viricidal activity. LB' can be replaced by H$_2$O$_2$, but not by non-H$_2$O$_2$-producing LB, and viricidal activity is inhibited by azide and catalase. The survival of HIV in the female genital tract and thus the likelihood of transmission may be influenced by the activity of the LB'--peroxidase-halide system in the vagina.

Heterosexual transmission of HIV is occurring with increasing frequency in the United States (1) and remains the most prevalent mode of transmission in Africa (2, 3). In heterosexual transmission, HIV released into the vagina by either partner survives the local defense mechanisms to infect sexual partners. However, the efficiency of sexual transmission is extremely variable, suggesting that there may be factors that can affect the survival of HIV in the vagina.

Peroxidases, when combined with H$_2$O$_2$ and a halide, form a powerful antimicrobial system which is effective against a variety of microorganisms (4), including viruses (5). Mammalian peroxidases effective in this way include myeloperoxidase (MPO) present in neutrophils and monocytes, eosinophil peroxidase (EPO) in eosinophils, and lactoperoxidase (LPO) in milk and saliva. A peroxidase with antimicrobial (6) and spermicidal (7) properties is also present in the uterine fluid of estrogen-primed rats. Although peroxidases are generally effective with several halides, the physiologic halide for MPO is believed to be chloride (4), for EPO bromide (8, 9) or the pseudohalide thiocyanate (10), and for LPO thiocyanate (11, 12). The source of H$_2$O$_2$ for the peroxidase-mediated antimicrobial system includes the respiratory burst of stimulated phagocytes and the metabolism of certain bacteria.

Bacteria designated as lactic acid bacteria, e.g., lactobacilli, streptococci and pneumococci, release H$_2$O$_2$ (13) which can be autoinhibitory (13) and, when mixed cultures are employed, can be toxic to other bacteria (6, 14), fungi (14), viruses (15), spermatozoa (16), or tumor cells (17), particularly in the presence of peroxidase and a halide. The ability of lactobacilli to provide H$_2$O$_2$ for a peroxidase-dependent cidal system is pertinent to the female genital tract, since the predominant bacterial species in normal human vaginal secretions is *Lactobacillus acidophilus*, and the vaginal overgrowth of a number of organisms in bacterial vaginosis is associated with a decrease in H$_2$O$_2$-generating lactobacilli (18). Thus, the production of H$_2$O$_2$ by lactobacilli in the vagina appears to be a nonspecific host defense mechanism which can be potentiated by peroxidases of leukocytic or uterine origin. In this study, we demonstrate the viricidal effect of the peroxidase-H$_2$O$_2$-halide system on HIV, and the ability of H$_2$O$_2$-generating lactobacilli to provide the H$_2$O$_2$ required for this effect.

**Materials and Methods**

**Human Immunodeficiency Virus.** HIV-1 (strain LAV-1 kindly provided by Genetic Systems, Seattle, WA) was propagated in CEM cells (kindly provided by Genetic Systems) in CEM growth medium (RPMI-1640 [Gibco Laboratories, Grand Island, NY] containing 10% Fetal Bovine Serum [Gibco Laboratories], 50 U/ml penicillin, 50 mcg/ml streptomycin, 0.01% DEAE Dextran [500,000 Mr; Sigma Chemical Co., St. Louis, MO], and 0.01 M Hepes buffer, pH 6.8). HIV in the cell supernatant was diluted to 10$^8$ tissue culture infective dose 50 (TCID$_{50}$/ml in CEM growth medium and frozen in liquid nitrogen gas phase. Stock virus was diluted 100-fold with 0.1 M sodium sulfate prior to a further 10-fold dilution in the reaction mixture (final concentration, 1000 TCID$_{50}$/ml). In addition to the components listed, the reaction mixture contained the components of CEM growth medium diluted 1,000-fold.

**Peroxidase.** MPO was purified from human leukocytes (19) and...
Vericidal Effect of Lactobacillus acidophilus

EPO from horse eosinophils (20). Peroxidase activity was determined by guaiacol oxidation (21).

Lactobacilli. Hospital cultures of L. acidophilus obtained from human vaginal swabs were initially tested for H2O2 production by exposure to air of colonies which had been grown aerobically as surface colonies on agar containing horseradish peroxidase and tetramethylbenzidine (18). Colonies of H2O2-generating organisms (LB+) formed a blue pigment, whereas organisms which did not form H2O2 (LB-) remained white. LB+ and LB- cultures were maintained by aerobic growth on HBT agar plates (01-478 Remel, Lenexa, KS) at 37°C in a CO2 incubator for no longer than 2 d. Before the experiment, the organisms were transferred to peptone yeast extract medium (22) containing 1% heat-inactivated FCS (HyClone Laboratories, Logan, UT) and grown aerobically at 37°C for approximately 6 h with tumbling for LB+ and 24 h in stationary tubes for LB-. The lactobacilli were washed twice and suspended in 0.1 M sodium sulfate. H2O2 generation by LB+ was determined before each experiment by the scopoletin method (23).

Measurement of Viricidal Activity. The reaction mixtures described in the legends to the figure and table were incubated in sterile screwcap microtubes with sealing O-rings (4.3 x 10.8 mm, 1.5 ml capacity #75.692.005; Sarstedt, Inc., Princeton, NJ) for 30 min at 37°C. The tubes were centrifuged at 2,940 x g for 2 min to sediment the bacteria and 10 μl aliquots of the supernatant fraction were added to triplicate wells (48-well plate #3548; Costar, Cambridge, MA) containing 2 x 105 CEM cells in 1 ml of CEM grown medium per well. Following incubation at 37°C for 6 d in a CO2 incubator (5% CO2-95% air), a 200 μl aliquot was removed for measurement of HIV P24 antigen by a solid phase sandwich-type ELISA (Abbott Laboratories, Chicago, IL). If an optical density of >2.000 was observed, the supernatant fraction was diluted and remeasured. The data are expressed as the P24 antigen level above background. A positive HIV-P24 antigen cutoff of 10 pg/ml was used. In general, culture supernatant levels of HIV P24 antigen were either zero (<10 pg/ml), indicating no replication, or very high (>1,000,000 pg/ml), indicating unrestricted replication, with the level reached being related to the number of CEM cells (2 x 109), the volume of the reaction mixture added to the CEM cells (10 μl) and the growth period (6 d).

Results and Discussion

H2O2-generating L. acidophilus (LB+) alone at a concentration of 104 CFU/ml was viricidal to HIV in lactate buffer pH 5.0 as measured by a decrease in viral replication in CEM cells (Fig. 1). When the LB+ concentration was reduced to a level where it was ineffective alone (2 x 105-5 x 106 CFU/ml), the addition of MPO and chloride resulted in a return of HIV viricidal activity (Fig. 1).

The HIV viricidal activity of high concentrations of LB+ alone was variable, with complete loss of HIV infectivity observed in five of nine wells, giving a median P24 level of <10 and a mean of 123 pg/ml (Table 1). An anti-HIV effect was not seen when the LB+ was heated at 100°C for 15 min or was replaced by L. acidophilus which did not generate H2O2 (LB-). This toxic effect on HIV was inhibited by catalase, but not by heated catalase, implicating H2O2. Table 1 also demonstrates the viricidal effect of low levels of LB+ combined with MPO and chloride, the requirement for each component of the system, and the inhibition by heat-treatment of LB+, by the substitution of LB- for LB+ and by the addition of azide (which inhibits peroxidase) or catalase, but not heated catalase or superoxide dismutase, again implicating H2O2. Chloride could be replaced by iodide, bromide or thiocyanate ions, LB+ could be replaced by reagent H2O2 and MPO could be replaced by EPO. However, chloride was less effective with EPO than with MPO.

The inhibition of HIV infectivity was not due to direct toxicity to the CEM cells. The evidence is as follows: (a) the reaction mixture was diluted 100-fold on addition to the CEM cells; (b) the CEM cells continue to metabolize and divide at a normal rate as indicated by acid production and increase in cell number; (c) identical results were observed when catalase was used to stop the reaction before the addition of an aliquot to the CEM cells; and (d) the toxicity of the peroxidase system is known to be strongly inhibited by protein and low mol wt components present in the CEM growth medium.

L. acidophilus has been detected in the vagina of most normal women with the level of H2O2-producing lactobacilli averaging 8.4 x 108 organisms per ml in one study (18). Under our in vitro conditions, the amount of H2O2 generated by 105 organisms was sufficient to inactivate HIV in the absence of peroxidase, and when MPO and chloride were added, concentrations of LB+ as low as 2 x 105 CFU/ml were effective. Thus, lactobacilli appear to be present in the vagina in adequate numbers to exert an antiviral effect. Peroxidase has also been detected in human vaginal fluid (24). Of the halides, chloride is present in cervical mucus in high concentration (25) and iodide and thiocyanate are concentrated there following intravenous administration to humans (26). Their presence in vaginal fluid would thus be anticipated. The pH employed here is approximately that of vaginal fluid. Additional studies are needed to evaluate the influence of potential inhibitors in vaginal fluid (catalase, protein, low mol wt-reducing substances).
Table 1. Properties of the LB+-Dependent Human Immunodeficiency Virus (HIV)-Inhibitory System

| Additions                        | HIV P24 antigen (pg/ml) | No. with HIV growth/No. of samples |
|----------------------------------|-------------------------|------------------------------------|
| None                             | 1,202,604               | 9/9                                |
| LB+ (10^6/ml)                    | 123                     | 4/9                                |
| LB+ heated                       | 2,063,677               | 3/3                                |
| LB+ deleted, LB- added           | 1,982,759               | 3/3                                |
| Catalase added                   | 162,754                 | 8/9                                |
| Heated catalase added            | 198                     | 5/9                                |
| LB+ (10^6/ml) + MPO + CI         | <10                     | 0/12                               |
| Cl deleted                       | 2,303,638               | 3/3                                |
| MPO deleted                      | 4,368,805               | 3/3                                |
| LB- deleted                      | 3,760,265               | 3/3                                |
| LB+ heated                       | 5,614,311               | 3/3                                |
| LB+ deleted, LB- added           | 3,685,807               | 3/3                                |
| Azide added                      | 3,368,574               | 3/3                                |
| Catalase added                   | 5,075,827               | 3/3                                |
| Heated catalase added            | <10                     | 0/6                                |
| SOD added                        | <10                     | 0/6                                |
| Cl deleted, I added              | <10                     | 0/6                                |
| Cl deleted, Br added             | <10                     | 0/6                                |
| Cl deleted, SCN added            | <10                     | 0/6                                |
| LB+ deleted, H2O2 added          | <10                     | 0/6                                |
| LB+ (10^6/ml) + EPO + CI         | 12,965                  | 6/6                                |
| Cl deleted, I added              | <10                     | 0/6                                |
| Cl deleted, Br added             | <10                     | 0/6                                |
| Cl deleted, SCN added            | <10                     | 0/6                                |

We have concentrated on the destruction of cell-free HIV. HIV in semen is both cell-associated and free in seminal plasma (27) and it is not clear which form is responsible for male to female vaginal transmission. The infection of primates by intravaginal infusion of cell-free HIV or SIV, however, has been described (28, 29).

Our findings suggest that the presence and level of H2O2-producing lactobacilli in vaginal fluid may affect the heterosexual transmission of HIV by the release of H2O2, which can act either alone or in conjunction with a halide and peroxidase of leukocytic or uterine origin to inactivate HIV. Normal individuals, or persons with sexually transmitted vaginal disease, in whom vaginal H2O2-producing lactobacilli are few or absent, may thus be at greater risk of infection and, as a corollary, may benefit from vaginal colonization with H2O2-generating lactobacilli. The systemic treatment of AIDS patients with orally-administered live lactobacilli has been proposed (30). The presence of peroxidase in other locations, e.g., LPO in milk and saliva, MPO in neutrophils and monocytes, EPO in eosinophils and extracellular MPO or EPO in inflammatory loci containing degenerating or stimulated phagocytes, raise the possibility that the peroxidase system utilizing H2O2 formed by microorganisms, phagocytes or soluble enzymes also contributes to the host defense against HIV at other sites.

The reaction mixture was as described in Fig. 1, except that the additions were: LB+ and LB- at the concentrations indicated; 23 mU/ml MPO; 23 mU/ml EPO; 0.1 M sodium chloride; 10^-4 M sodium bromide; 10^-4 M sodium thiocyanate; 10^-5 M H2O2; 10^-4 M sodium azide; 5.8 hg/ml catalase (CTR bovine liver, 84,150 U/mg, [Worthington Biochem, Freehold, NJ] dialyzed before use); 10 μg/ml superoxide dismutase (SOD, bovine erythrocyte, 3,150 U/mg, [Sigma Chemical Co.]). The LB+ and catalase were heated for 15 min at 100°C where indicated. The heated LB+ was collected by centrifugation and resuspended in 0.1 M sodium sulfate to the required optical density. The results are expressed as the mean 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. The results are the mean of 3-9 determinations.

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References

1. Holmes, K.K., J.M. Karon, and J. Kreiss. 1990. The increasing frequency of heterosexually acquired AIDS in the United States, 1983–88. Am. J. Public Health. 80:858.

2. Quinn, T.C., J.M. Mann, J.W. Curran, and P. Piot. 1986. AIDS in Africa: an epidemiologic paradigm. Science (Wash. DC). 234:955.

3. Havlikos, H.W., and R. Edelman. 1988. The epidemiology of Acquired Immuno deficiency Syndrome among heterosexuals. JAMA (J. Am. Med. Assoc.). 260:1922.

4. 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. pp. 391.

5. Belding, M.E., S.J. Klebanoff, and C.G. Ray. 1970. Peroxidase-mediated virucidal systems. Science (Wash. DC). 167:195.

6. Klebanoff, S.J., and D.C. Smith. 1970. Peroxidase-mediated antimicrobial activity of rat uterine fluid. Gynecol. Invest. 1:21.

7. Smith, D.C., and S.J. Klebanoff. 1970. A uterine fluid-mediated sperm inhibitory system. Biol. Reprod. 3:229.

8. Weiss, S.J., S.T. Test, C.M. Eckmann, R. Ross, and S. Regiani. 1986. Brominating oxidants generated by human eosinophils. Science (Wash. DC). 234:200.

9. Mayeno, A.N., A.J. Curran, R.L. Roberts, and C.S. Foote. 1989. Eosinophils preferentially use bromide to generate halogenating agents. J. Biol. Chem. 264:5660.

10. Slungaard, A., and J.R. Mahoney, Jr. 1991. Thiocyanate is the major substrate for eosinophil peroxidase in physiologic fluids: implications for cytotoxicity. J. Biol. Chem. 266:4903.

11. Reiter, B., A. Pickering, and J.D. Oram. 1964. An inhibitory system - lactoperoxidase/thiocyanate/peroxide - in raw milk. 4th Intl. Symp Food Microbiol. SIK. Goteborg, Sweden. pp. 297.

12. Dogon, I.L., A.C. Kerr, and B.H. Arndt. 1962. Characterization of an antibacterial factor in human parotid secretions, active against Lactobacillus casei. Arch. Oral. Biol. 7:81.

13. Klebanoff, S.J., and R.A. Clark. 1978. The Neutrophil: Function and Clinical Disorders. North-Holland Publishing, Amsterdam. pp. 414–416.

14. Hamon, C.B., and S.J. Klebanoff. 1973. A peroxidase-mediated Streptococcus mitis-dependent antimicrobial system in saliva. J. Exp. Med. 137:438.

15. Klebanoff, S.J., and M.E. Belding. 1974. Virucidal activity of H2O2-generating bacteria: requirement for peroxidase and a halide. J. Infect. Dis. 129:345.

16. Klebanoff, S.J., and D.C. Smith. 1970. The source of H2O2 for the uterine fluid-mediated sperm-inhibitory system. Biol. Reprod. 3:236.

17. Clark, R.A., S.J. Klebanoff, A.B. Einstein, and A. Fefer. 1975. Peroxidase-H2O2-halide system: cytotoxic effect on mamalian tumor cells. Blood. 45:161.

18. 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.

19. 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.

20. Jorg, A., J.M. Pasquier, and S.J. Klebanoff. 1982. Purification of horse eosinophil peroxidase. Biochim. Biophys. Acta. 701:185.

21. Klebanoff, S.J., A.M. Waltersdorph, and H. Rosen. 1984. Antimicrobial activity of myeloperoxidase. Methods Enzymol. 105:399.

22. Holdman, L.V., E.P. Cato, and W.E.C. Moore. 1977. Anaerobe laboratory manual. VPI Anaerobe Laboratory, Blacksburg, Virginia. pp. 144.

23. Root, R.K., J. Metcalf, N. Oshino, and B. Chance. 1975. H2O2 release from human granulocytes during phagocytosis. Document, quantitation, and some regulating factors. J. Clin. Invest. 55:945.

24. 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.

25. Herzberg, M., C.A. Joel, and A. Katchalsky. 1964. The cyclic variation of sodium chloride content in the mucus of the cervix uteri. Fertil. Steril. 15:684.

26. von Kaula, K.N., J.K. Aikawa, P.D. Bruns, W.T. Wikle, and V.E. Drose. 1957. H2O2 release from human granulocytes during phagocytosis. J. Acq. Immune Def Synd. 1:419.

27. Borzy, M.S., R.S. Connell, and A.A. Kiessling. 1988. Detection of human immunodeficiency virus in cell-free seminal fluid. J. Virology. 63:4277.

28. Miller, C.J., N.J. Alexander, S. Sutjipto, A.A. Lackner, A. Gettie, A.G. Hendricks, L.J. Lowenstein, M. Jennings, and P.A. Marx. 1989. Genital mucosal transmission of simian immunodeficiency virus: animal model for heterosexual transmission of human immunodeficiency virus. J. Infect. Dis. 154:896.

29. Miller, C.J., N.J. Alexander, S. Sutjipto, A.A. Lackner, A. Gettie, A.G. Hendricks, L.J. Lowenstein, M. Jennings, and P.A. Marx. 1989. Genital mucosal transmission of simian immunodeficiency virus: animal model for heterosexual transmission of human immunodeficiency virus. J. Virology. 63:4277.

30. Tihole, F. 1988. Possible treatment of AIDS patients with live lactobacteria. Med. Hypotheses. 26:85.