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Effects of Varying Concentrations of Bleach on in vitro HIV-1 Replication and the Relevance to Injection Drug Use

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Abstract
The use of bleach (hypochlorite) as a disinfectant for drug injection equipment in the intravenous-drug-using population was recommended early in the HIV-1/AIDS epidemic. Epidemiological studies have challenged the use of bleach as an effective measure to prevent HIV-1 transmission. However, in vitro HIV-1 coculture studies have shown that a high concentration of bleach is an effective cytotoxic and potentially virucidal agent. In this study, we demonstrate that HIV-1 peripheral blood mononuclear cell cocultures containing low concentrations of hypochlorite in the media showed earlier conversion to HIV-1 positivity, as measured by the presence of p24 antigen. HIV-1 cocultures with high concentrations of hypochlorite in the culture media, which appeared to be highly cytotoxic, and HIV-1 cocultures without bleach in the media did not exhibit this early p24 antigen positivity. Hypochlorite chemically disinfects by releasing free chlorine that is a potent oxidant. In injection drug equipment, a low residual concentration of bleach is likely to remain in cleaned equipment despite rinsing with water. Low concentrations of oxidants have been shown to enhance tissue inflammation, in vivo, as well as HIV-1 replication in vitro. Previous studies have shown that despite vigorous cleaning of blood-contaminated injection syringes with bleach followed by water, microaggregates of residual blood remained in bleach-cleaned blood-contaminated syringes. Hypothetically, oxidant effects of the residual bleach in the bleach-cleaned syringes could enhance the possibility of infection by remaining HIV-1 contained in a contaminated syringe. We suggest that the likelihood of an injection drug user contracting HIV-1 through the sharing of a bleach-cleaned blood-contaminated syringe may be increased by the cotransmission of residual bleach and its localized tissue-inflammatory effects; however, this has not been statistically proven in epidemiological studies.

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Introduction

The use of bleach as an HIV disinfection agent for injection drug users is controversial; however, bleach cleaning prior to sharing or reusing syringes and other injection equipment has been a cornerstone of outreach programs. These practices are performed in an attempt to reduce the transmission of HIV-1 and other parenteral diseases such as HTLV, hepatitis B and C among injection drug users [1–3]. In the current study, we sought to examine the effects of increasing concentrations of bleach on HIV-1 peripheral blood mononuclear cell (PBMC) coculture to determine the bleach concentrations that would inhibit HIV-1 propagation, as determined by p24 antigen detection. The relevance of these studies to syringe cleaning by bleach disinfection and injection drug use is discussed.

Methods

HIV Culture Techniques

Qualitative cell cultures in replicates of 6 per test condition (42 total) were evaluated. Eight milliliters of culture medium was prepared containing 1.0×10^7 target uninfected PBMCs. 8.0×10^5 infected frozen PBMCs, containing 100 in vitro HIV-1 infectious units, were washed and inoculated into each culture flask [4]. Common household bleach (5.25%) obtained from a local grocery store was diluted with deionized water in serial 1:5 dilutions to give the following hypochlorite concentrations by volume: 1.0, 0.1, 0.01, 0.001 and 0.0001%. The hypochlorite was diluted into 10 ml of tissue culture medium, to obtain the final desired concentrations. Neither bleach nor HIV-infected PBMCs were added to the negative control flask. The positive control and test flasks had 100 units of HIV-1-infected PBMCs without the addition of diluted bleach. All flasks were incubated at 37°C and 95% humidity. A baseline p24 antigen assay was performed on 0.5 ml of culture supernatant collected after 2 h of incubation. Target cell viability estimation was performed using trypan blue staining and visual observation of the cells under an inverted microscope. On days 0, 3, 7 and 14, viability measures were obtained. The cultures were fed on day 3 with 4 ml of fresh tissue culture media containing hypochlorite, and thereafter (on day 7 and subsequent days) with 4 ml of fresh tissue culture media which did not contain hypochlorite. Samples of tissue culture media were collected for the p24 antigen assay immediately before the addition of fresh tissue culture medium.

HIV p24 Antigen Assay

p24 antigen assays were performed using the p24 ELISA kit (Abbott Laboratories, Inc. Chicago, Ill., USA), per the manufacturer's instructions, in order to assess HIV-1 replication. The minimum detectable p24 antigen level for this kit was approximately 30 pg/ml. HIV-1-positive cultures were defined as having a fourfold, or greater, increase in the p24 antigen concentration using serial consecutive measures of the cell culture media collected during the course of the study.

Table 1. Percent HIV-1 culture target cell viability as measured by direct inverted microscopy

	0 days	3 days	7 days	14 days
1.0% bleach	49%[1]	48%[1]	36%[1]	95%
0.1% bleach	48%[1]	45%[1]	20%[1]	93%
0.01% bleach	99%	100%	98%	95%
0.001% bleach	99%	99%	98%	95%
0.0001% bleach	100%	100%	100%	95%
HIV-1– control	98%	99%	98%	98%
HIV-1+ control	98%	100%	100%	97%

Cell counts are obtained on one flask at each concentration; if target cell viability on the first flask is determined to be less than 80% then the second flask is counted. If any flask is determined to be less than 50% then each of the six flasks replicates is counted.

[1] Value represents the mean of actual cell count for the six replicates at time point.

Detection of Total and Free Chlorine

Free and total chlorine concentrations, after dilution into the cell culture media, were assessed using a Hacke Test Kit™ Model cn/66/66F/66T. The range of detection of both free and total chlorine using this kit is 0–3.5 mg/ml. Calibration was performed using a fresh chlorine reagent, and the culture medium samples containing various hypochlorite concentrations were compared to the standards.

Results

The HIV-1 PBMC coculture system was chemically inactivated by treatment with high concentrations of bleach, and the minimal bleach concentrations which were inhibitory to virus production were determined by HIV-1 p24 antigen detection. Various concentrations of bleach were tested on uninfected human PBMCs in culture to determine the effects of household bleach on the viability of PBMCs alone. The concentrations of bleach in culture were verified using the Hacke Kit. Table 1 shows a marked cytotoxic effect of bleach on target cell viability at the two highest hypochlorite concentrations. This toxic effect increased during the first week of incubation, with viable cells decreasing from 49 to 36% at the highest hypochlorite concentration (1.0%). The number of viable cells after 7 days in culture dropped from 48 to 20% at the 0.1% hypochlorite concentration. The three lowest hypochlorite concentrations appeared to have no significant effects on cellular viability when compared to the HIV-1-negative and -positive control cultures. Replacement of

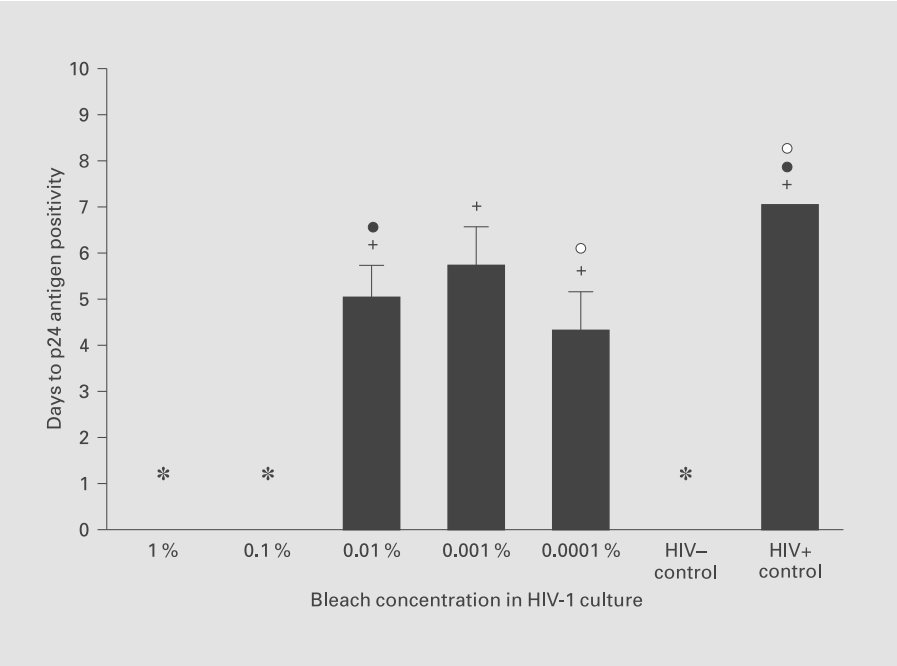

Fig. 1. This figure shows the mean (± SE) number of days required for the 6 replicate bleach cultures to become p24 positive (>30 pg/ml). There are significant differences when compared with the HIV-positive control cultures (n = 6) for the individual bleach concentrations as noted. * p = The two highest bleach concentrations 1% (n = 6) and 0.1% (n = 6) as well as the HIV-negative control cultures (n = 6) showed [p24] <30 pg/ml serially over 28 days in culture; ● = 0.01% bleach concentration (n = 6; p = 0.09) compared with HIV-positive control; ○ = 0.0001% bleach concentration (n = 6; p = 0.033) compared with HIV-positive + control; + = pooled results for bleach concentrations 0.01, 0.001 and 0.0001 (n = 18; p = 0.034) compared with HIV+ control.

the bleach-containing media with fresh non-hypochlorite-containing media, at day 7, appeared to enable cell replication, as target cell viabilities at day 14 were all greater than 90%.

Figure 1 demonstrates the number of days of culture required for the HIV-1-positive control and bleach-exposed HIV-1-infected cultures to become p24 antigen positive. The cultures with the two highest hypochlorite concentrations (1.0 and 0.1%) failed to exhibit a positive p24 response, most likely due to chemical inactivation. However, the cultures containing the three lowest hypochlorite concentrations (0.01, 0.001 and 0.0001%, respectively) were determined to be p24 antigen positive after 5.0, 5.7 and 4.3 days of incubation, respectively. The mean number of days of incubation required for the HIV-1-positive control culture, containing no bleach, to be determined as p24 antigen positive was 7 days.

The earlier occurrence of p24 antigen positivity in HIV-1-infected cultures containing the three lowest concentrations of hypochlorite, when compared to the HIV-1-positive control culture without bleach was an unexpected result. A one-tailed Mann-Whitney exact test was used initially to determine the statistical significance of the numbers of bleach-exposed cultures that converted to p24 antigen positivity earlier than the HIV-1-positive control cultures. Due to the small sample size per group, a significance level of $p < 0.1$ was chosen to reduce the probability of type II errors. When the individual hypochlorite concentration groups were tested against the HIV-1-positive control, the 0.01 and 0.0001% concentrations were statistically significantly different from the positive control, with p = 0.09 and p = 0.033, respectively. The 0.001% hypochlorite concentration group converted to p24 antigen positivity earlier than the HIV-1-positive control culture; however, the conversion time was not statistically significantly different from that of the positive control. The twice-weekly fixed schedule for p24 testing of culture supernatant used in this culture protocol introduced the possibility for random fluctuations in the detected p24 antigen values. When the data from the cultures containing the three lowest hypochlorite concentrations were combined, as an adjustment for the possibility

of random fluctuations, the combined earlier times of appearance of p24 antigen were determined to be statistically significantly different with $p = 0.034$.

Discussion

This study shows that high concentrations of bleach (hypochlorite) inhibit HIV-1 replication in vitro, as evidenced by cultures to produce p24 antigen production by HIV-1-infected PBMCs. As expected, high concentrations of bleach reduced culture target cell viability, as well as HIV-1 p24 antigen production. An unexpected finding was that lower concentrations of bleach in the cell culture medium, however, did not reduce target cell viability and appeared to be permissive for HIV-1 infection and replication in cell cultures. These observations may be relevant to injection drug use and bleach cleaning of contaminated injection equipment. In addition this observation may be important to other in vitro studies which have been performed to establish the effectiveness of bleach as a disinfectant for contaminated injection equipment [5–9].

The range of lower residual bleach concentrations likely represents the concentration of bleach retained in syringes after recommended bleach washing (two full-strength bleach washes followed by two water rinses). If this finding is paired with previous experiments, that show microaggregates of clotted blood from bleach-rinsed blood-containing syringes were observed and residual contaminating DNA from blood and HIV-1 was easily detected using PCR technology [11]. It is possible that low concentrations of bleach coinjected with microaggregates of HIV-1-infected blood might enhance the infectivity of HIV-1 in the drug injector.

A possible mechanism for the permissive effects of low bleach concentrations on HIV-1 replication in vitro, and potentially in vivo, is oxidative effects of free chlorine derived from the bleach. Previous studies have shown that oxidative stressors accelerate cellular injury [14–17], mediate cytokine secretion, and enhance local tissue inflammatory responses in HIV-1 infection [18]. In addition, adding the antioxidant, N-acetyl-*L*-cysteine to HIV-1 cultures has been shown to inhibit oxidative effects on in vitro HIV replication [19].

Theoretically, in an injection drug user, this prooxidant effect, mediated by free-radical formation from residual hypochlorite generated from low concentrations of bleach, in combination with the injection of drugs and trapped virions, may result in enhanced cellular and tissue susceptibility to HIV-1 infection. These biological factors as well as behaviorally related syringe-cleaning compliance issues may help to further explain the failure of bleach washing of injection equipment to be more protective in preventing HIV-1 infection [8, 9]. A more thorough clinical investigation of the role of disinfectants, as well as assessment of bleach involvement in inflammation at the injection site, in injection drug users, will be necessary to fully realize the in vivo significance of the in vitro HIV-1 coculture observations found in this study.

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References

1 Garfein RS, Vlahov D, Galai N, Doherty MC, Nelson KE: Viral infections in short-term injection drug users: The prevalence of the hepatitis C, hepatitis B, human immunodeficiency, and human T-lymphotropic viruses. Am J Public Health 1996;6:655–661.

2 Tenant M: Seroprevalence of hepatitis ABCD markers and liver function abnormalities in intravenous heroin addicts. J Addict Dis 1995; 14:35–49.

3 Alter MJ, Mast EE: The epidemiology of viral hepatitis in the US. Gastroenterol Clin North Am 1994;23:437–455.

4 Hollinger FB (ed): Quantitative PBMC microculture assay; in Virology Manual for HIV Laboratories. Version 2.1. Sponsored by the Division of AIDS, National Institute on Allergic and Infectious Diseases, National Institutes of Health, Bethesda MD, 1993;5–11. ACTG protocols for HIV culture.

5 Flynn NS, Jain EM, Keddie EM, Carlson JR, Jennings MB, Haverkos HW, Nassar N, Anderson R, Cohen S, Goldberg D: In vitro activity of readily available household materials against HIV-1: Is bleach enough? J Acquir Immune Defic Syndr Hum Retrovirol 1994;7:747–753.

6 Shapshak PC, McCoy CB, Shah SM, Page JB, Rivers JE, Weatherby NL, Chitwood DD, Mash DC: Preliminary laboratory studies of inactivation of HIV-1 in needles and syringes containing infected blood using undiluted household bleach. J Acquir Immune Defic Syndr Hum Retrovirol 1994;7:754–759.

7 Gleghorn AA, Doherty MC, Vlahov D, Celentano DD, Jones TS: Inadequate bleach contact times during syringe cleaning among injection drug users. J Acquir Immune Defic Syndr Hum Retorvirol 1994;7:767–772.

8 Vlahov D, Astemborski J, Solomon L, Nelson KE: Field effectiveness of needle disinfection among injecting drug users. J Acquir Immune Defic Syndr Hum Retrovirol 1994;7:760–766.
9 McCoy CB, Rivers JE, McCoy HV, Shapshak P, Weatherby NL, Chitwood DD, Page JB, Inciardi JA, McBride DC: Compliance to bleach disinfection protocols among protocols among injecting drug users in Miami. J Acquir Immune Defic Syndr Hum Retrovirol 1994;7:773–776.
10 Centers for Disease Control. Update: Acquired immunodeficiency syndrome and human immunodeficiency virus among health-care workers. Mor Mortal Wkly Rep 1988;37:229–239.
11 Contoreggi CS, Jones SW, Simpson PM, Lange WR, Meyer WA III: A model of syringe disinfection as measure by polymerase chain reaction for human leukocyte antigen and HIV genome. 8th International Conference on AIDS, Amsterdam, 1992.
12 Newmeyer JA: Why bleach? Fighting AIDS contagion among intravenous drug users. San Francisco Psychoactive Drugs 1988;20:159–163.
13 Watters JK: Historical perspectives on the use of bleach in HIV/AIDS prevention. J Acquir Immune Defic Syndr Hum Retrovirol 1994;7:743–747.
14 Weiss SJ: Tissue destruction by neutrophils. N Engl J Med 1989;320:365–376.
15 Aust SD, Morehouse LA, Thomas CE: Role of metals in oxygen radical reactions. J Free Radic Biol Med 1985;1:3–25.
16 Sevenian A, Hochstein P: Mechanisms and consequences of lipid peroxidation in biological systems. Annu Rev Nutr 1985;5:365–375.
17 Scholes RG: Radiation effects on DNA. Br J Radiol 1983;56:221–231.
18 Baier-Bitterlich G, Fuch D, Wachter H: Chronic immune stimulation, oxidative stress, and apoptosis in HIV infection. Biochem Pharmacol 1997;53:755–763.
19 Roederer M, Staal FJ, Raju PA, Ela SW, Herzenberg LA: Cytokine stimulated human immunodeficiency virus replication is inhibited by N-acetyl-*L*-cysteine. Proc Natl Acad Sci USA 1990;87:4884–4888.