Survival of Human Immunodeficiency Virus (HIV), HIV-Infected Lymphocytes, and Poliovirus in Water

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The potential for human immunodeficiency virus (HIV) to enter domestic sewers via contaminated body fluids such as blood has spurred interest in the survival of this virus in water and wastewater. This study focused on establishing the inactivation of HIV and productively infected lymphocytes in dechlorinated tap water. In addition, HIV survival was compared with that of poliovirus. Results indicated that either free HIV or cell-associated HIV was rapidly inactivated, with a 90% loss of infectivity within 1 to 2 h at 25°C and a 99.9% loss by 8 h. In comparison, poliovirus showed no loss of infectivity over 24 h. The presence of human serum in tap water slowed the rate of HIV inactivation through 8 h but did not stabilize the virus through 24 h. In addition, blood from stage IV AIDS patients was introduced into tap water, and the recovery of HIV was monitored by using both an infectivity assay and polymerase chain reaction amplification of viral sequences. Virally infected cells were no longer detectable after 5 min in dechlorinated tap water, while little diminution in amplifiable sequences was observed over 2 h. Thus, detection of viral sequences by polymerase chain reaction technology should not be equated with risk of exposure to infectious HIV.

Human immunodeficiency virus (HIV) is recognized as an etiologic agent epidemiologically associated with AIDS (2, 10, 13). Recognized routes of transmission include sexual contact, intravenous drug use, blood transfusion, blood exchange as a result of percutaneous injury, and infection of infants either pre- or postpartum from infected mothers (7). Neither household contact (9, 14, 19) nor vector-borne transmission (7) of HIV has been substantiated. Nonetheless, the potential for HIV to enter domestic sewers and, in turn, environmental waters through the introduction of discarded contaminated blood has raised questions as to the survival of the virus in water.

The amount of cell-free HIV in the blood of an infected individual remains controversial. HIV has been reported at levels as high as 1,000 to 10,000 tissue culture infective doses (TCID) per ml of plasma in recently infected individuals, which drop rapidly to 1 to 2 TCID/ml within a period of 2 to 4 weeks after the initial infection (8). During subsequent asymptomatic infection, HIV is frequently not recovered as free virus from sera, although levels of 10 to 100 TCID/ml have been reported (1, 6, 20). During later symptomatic disease stages, cell-free virus levels in plasma can again approach 1,000 to 10,000 TCID/ml (1, 6, 12, 20), although most patients have lower levels of viremia even in late-stage disease.

HIV is routinely recovered from infected individuals by stimulation and subsequent culture of peripheral blood mononuclear cells. The number of HIV-infected cells has been variously estimated to range from 1 in 107 to 1 in 109 depending upon the disease status of the infected individual (1, 8, 21, 22, 24). However, the number of circulating lymphocytes actively replicating HIV may be as much as 2 orders of magnitude lower than these estimates (11, 18).

No recovery of infectious HIV from any environmental water source has been reported. In one instance, the detection of nucleic acid sequences with homology to HIV was reported (17); however, these results are controversial. Two studies have evaluated the survival of HIV in water. Slade et al. (23) found a mean reduction of HIV infectivity ranging from −0.35 to −0.63 log/day when virus was added to sterilized sewage, drinking water, and seawater held at 16°C. More recently, Casson et al. (3) observed a 10-fold reduction in HIV infectivity between 6 and 12 h in sterile water, with a 100-fold loss after 48 h and a 1,000-fold loss at 72 h. In the study by Casson et al., HIV survival in either primary or nonchlorinated secondary effluents from a wastewater treatment plant was not significantly different.

These studies (3, 23) demonstrate that HIV can maintain its viability for a limited time in selected water environments. Indeed, the organic content of primary and secondary effluents does afford protection against viral inactivation. However, factors such as osmotic pressure and residual chlorine could play a role in the inactivation of viruses such as HIV when the viruses are introduced into tap water.

The purpose of the work described in this report was to evaluate the stability of cell-free HIV, lymphocytes actively replicating HIV, and poliovirus in tap water. In addition, the detection of HIV proviral DNA by polymerase chain reaction (PCR) amplification was compared with the recovery of HIV by an infectivity assay.

MATERIALS AND METHODS

Virus and cells. HIVpM233 has been described previously (5); it was grown and its titers were determined in the human CD4-positive T-cell line CEM (American Type Culture Collection). Cells were grown as suspension cultures in RPMI 1640 medium containing 5% fetal calf serum (FCS), 5% calf serum (CS), penicillin (100 U/ml), and streptomycin (100 µg/ml).

Poliovirus I (Chat) was grown and assayed as PFU in HeLa cell monolayers as described previously (15). Briefly, cell monolayers were inoculated in triplicate and, after 1 h of absorption, overlayed with Eagle’s minimum essential medium containing 5% CS and 1% agar. After 2 days of incubation at 37°C in 5% CO2 in air, cells were stained with neutral red in Eagle’s minimum essential medium and plaques were counted.
Peripheral blood lymphocytes (PBLs) from HIV-seronegative donors were isolated by standard density gradient banding with lymphocyte separation medium (Organon Teknika, West Chester, Pa.) as described in the manufacturer's instructions. PBLs were collected from the interface and diluted with 10 ml of Hank's balanced salt solution containing 2% CS. Lymphocytes were collected by low-speed centrifugation (500 x g) and resuspended in 10 ml of Hank's balanced salt solution to wash out any remaining lymphocyte separation medium. After a final centrifugation, PBLs were cultured in RPMI 1640 medium containing 15% FCS, antibiotics, and 2.5 ng of phytohemagglutinin (Sigma Chemical Co., St. Louis, Mo.) per ml and incubated at 37°C in 5% CO2 in air. Forty-eight hours later, culture medium was replaced with RPMI 1640 medium as described above but containing 2.5 ng of recombinant interleukin-2 (Immunex Corp., Seattle, Wash.) per ml in place of phytohemagglutinin. PBLs were infected with HIV at a multiplicity of infection of about 0.5 and monitored for viral production as described below beginning 3 days postinfection. PBLs were used for stability studies when about 10% of the cells were replicating HIV.

HIV infectivity assays. Viral titrations were performed in 96-well tissue culture plates containing approximately 104 CEM cells per well in 0.1 ml of RPMI 1640 medium. Viral inoculum diluted in 10-fold serial dilutions was added to each well, and additional culture medium was added 2 days later. HIV infection was monitored by indirect fixed-cell immunofluorescence by using monoclonal antibody (M26) to the HIV core protein p24. Cells were spotted onto printed slides, fixed with 80% cold acetone, and incubated with M26 and then with fluorescein isothiocyanate-conjugated goat antimouse antibody (Sigma). Stained cells were viewed with a Zeiss microscope equipped for fluorescein isothiocyanate detection. Alternatively, cells were acetone fixed in suspension, stained, and then scored for fluorescence with an EPICS Profile flow cytometer (Coulter Corp., Hialeah, Fla.). The highest viral dilution producing HIV after 4 weeks was recorded as the end-point titer.

To recover infectious HIV from the peripheral blood of AIDS patients, fluids or PBLs were cocultured with stimulated pooled donor PBLs from HIV-seronegative, PCR-negative individuals. Where indicated, a mixture of HIV-susceptible cell lines, including VB, Jurkat, and THP-1 (AIDS Research and Reference Program, National Institute of Allergy and Infectious Diseases, Bethesda, Md.), was used as target cells for HIV infectivity assays. These cell lines represent both T-cell and promonocytic cell lineages and provide a sensitive assay system for the recovery of clinical HIV variants. Culture supernatants were collected twice weekly, beginning about 1 week after culture initiation, and held at -70°C. After 3 to 4 weeks, aliquots from each sample were pooled and tested for p24 by using a commercially available antigen capture enzyme immunoassay (Coulter) as described in the manufacturer's instructions.

Stability studies. High-titer HIV stocks (greater than 106 TCID/ml) were prepared from supernatants collected at 24-h intervals from HIV-infected CEM cells. Virus was concentrated by ultracentrifugation (100,000 x g, 90 min), and pellets were resuspended in phosphate-buffered saline (PBS) containing 0.1 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-N,N',N'-N"-tetraacetic acid] to facilitate dispersion of virions and held overnight on ice for use the next day. Poliovirus was used directly from high-titer stock (greater than 108 PFU/ml).

Tap water, collected from laboratory faucets after 1 min of flushing, was dechlorinated by the addition of sodium thiosulfate to a final concentration of 0.1 mM and then held overnight to allow equilibration to 25°C. The specific conductance of tap water samples used in these experiments ranged from 400 to 480 μS/cm; the pH was from 6.5 to 7.0. Tap water osmolality, measured with a micro-osmometer (Precision Systems, Inc., Natick, Mass.), was indistinguishable from zero, while PBS osmolality was measured at 280 mosmol/kg.

HIV inactivation was monitored by adding 0.1 ml of virus inoculum to 0.9 or 4.9 ml of tap water (1:10 and 1:50 dilutions, respectively), vortexing the mixture for 10 s, and immediately removing samples for dilution into RPMI 1640 culture medium and subsequent inoculation into CEM cells as described above. Poliovirus was diluted 1:100 in tap water, resulting in an input concentration of about 106 PFU/ml.

For lymphocyte viability studies, cells were collected by centrifugation (500 x g, 5 min) and pellets were resuspended at a density of about 106 cells per ml in PBS or water. At indicated times, aliquots were removed directly into 0.2% trypan blue in Hank's balanced salt solution containing 2% CS. Total and viable cells were counted with a hemocytometer. For the isolation of cell-associated HIV from lymphocytes, samples were inoculated directly into wells containing susceptible cells or diluted appropriately in culture medium and then assayed.

Venous blood was collected from HIV-seropositive adults (Centers for Disease Control stage IV) by using sterile blood collection tubes containing lithium heparin (Sherwood Medical, St. Louis, Mo.). Volumes of blood ranging from 0.1 to 1.0 ml were added to 50-ml volumes of either PBS or dechlorinated tap water equilibrated to 25°C. At indicated times, 5-ml volumes were placed into centrifuge tubes containing 1 ml of FCS. Cells were collected by low-speed centrifugation (500 x g, 5 min). Supernatant volumes were assayed in toto for HIV by the addition of 1 ml of 5 x RPMI 1640 medium and a mixture of susceptible cell lines (VB, Jurkat, and THP-1) to a density of 2 x 106/ml. After 24 h, the overlying fluid was removed and replaced with RPMI 1640 medium. Cultures were monitered for the replication of HIV as described above. PBLs collected from this same 5-ml sample were resuspended in 0.5 ml of RPMI 1640 culture medium, one-half of each volume was cocultured with donor PBLs, and the remainder was cultured with the cell mixture as described above. DNA was recovered by placing 1 ml of sample in a tube containing 0.1 ml of CS. Subsequently, total DNA was isolated by using a standard procedure of proteinase K digestion, phenol-chloroform extraction, and ethanol precipitation (25).

PCR amplification of proviral DNA. HIV sequences within the long terminal repeat were targeted for amplification by using primers specific for nucleotides 491 to 510 and 637 to 656, resulting in a predicted product of 166 bp (primer 1, 5'-TCCTGGCACTAGGAGAAC; primer 2, 5'-CAGTTC CCTGTTCGGGCGC). As a control, cellular sequences lying within exon 4 of the actin gene were also amplified with a predicted PCR product of 421 bp (primer 1, 5'-GAGACCT TCAACACCCCGGAC; primer 2, 5'-AGGGCTGAGAGCT GCCTCA). To allow visualization of amplified products, one oligonucleotide primer of each pair was end labeled with 32P by using T4 polynucleotide kinase as described in published procedures (26).

Total cellular DNA extracted from a constant volume of PBS or water was amplified in the presence of nucleotides and Taq polymerase for 30 cycles by using a thermal cycler.
programmed for 94°C denaturation for 1 min, 55°C annealing for 1 min, and 72°C polymerization for 1 min (16). Predicted PCR DNA fragments were separated on 8% polyacrylamide gels along with appropriate DNA markers (123-bp ladder; Gibco-BRL, Gaithersburg, Md.). Amplified sequences were visualized by autoradiography.

RESULTS

Initial studies were undertaken to evaluate the survival of HIV in tap water held at 25°C. Water was dechlorinated to represent a worst-case situation. To provide a basis of comparison, poliovirus survival was evaluated in parallel. The results shown in Fig. 1A indicate that HIV was relatively stable in PBS. While a 10-fold reduction in the HIV titer in PBS was observed within 8 h at 25°C, further reduction in titer through 24 h was minimal. When HIV was suspended in dechlorinated tap water held at 25°C, its infectivity was reduced by 10-fold within 30 to 60 min and 1,000-fold by 8 h. The addition of 5% human serum slowed viral inactivation during the first 8 h, but no infectious HIV could be recovered from tap water with or without serum at 24 h by using an experimental system capable of monitoring a 10,000-fold loss of HIV infectivity. The presence of 2% human serum in tap water did not enhance HIV survival compared with that in tap water alone in one experiment (data not shown). In a parallel experiment, poliovirus was equally stable in both PBS and dechlorinated tap water held at room temperature (Fig. 1B). No loss of infectivity was noted for this enteric virus over 24 h.

The fate of human cells introduced into tap water was first evaluated by using a transformed T-cell line, CEM. Upon introduction of CEM cells into tap water, cell viability was exceedingly limited. As shown in Fig. 2A, fewer than 5% of CEM cells remained viable within 5 min after introduction into tap water. In comparison, PBLs required about 15 min of exposure to reach a comparable loss (Fig. 2B). Therefore, subsequent experiments using PBLs were conducted. As shown in Fig. 2B, the addition of 2% human serum to dechlorinated tap water did little to change the viability profile of PBLs. However, the addition of 5% serum enhanced the stability of lymphocytes, allowing the survival of about 25 to 30% of the cell population. Since PBLs from two different donors as well as pooled PBLs representing three individuals were used in these experiments, it is likely that the surviving cells represented a subset of lymphocytes. The loss of both HIV and lymphocyte viability could be
attributed to the unfavorable osmotic pressure of tap water in comparison with that of isotonic fluids such as PBS. In separate experiments, the osmolality of these suspending media were compared. Values for dechlorinated tap water could not be distinguished from 0; the addition of human serum at final concentrations of 2 and 5% (vol/vol) resulted in osmolality readings of 6 and 12 mosmol/kg of water, respectively. Since human plasma has an osmotic pressure of about 290 mosmol/kg, these experimental values for human serum in tap water (1:50 and 1:20 dilutions) are comparable to predicted values.

To address the fate of HIV-infected lymphocytes in water, pooled PBLs from seronegative donors were infected in vitro and then used in stability tests. As observed for uninfected lymphocytes, cell viability was rapidly lost within 15 min in tap water (Fig. 3A). In this experiment, the presence of 2% serum slowed cell death somewhat; however, less than 10% of cells remained viable at 30 min. The reduction of viral infectivity associated with PBLs resembled the inactivation of free virus, with 10-fold loss within 1 h of tap-water exposure. By 8 h, a 100-fold loss of HIV infectivity was recorded in tap water. The presence of human serum enhanced cell-associated virus survival, with only a 90% reduction of infectivity observed at 8 h; however, no infectious virus was recovered from tap water with or without serum at 24 h. It should be noted that the assay used in this experiment could detect a 1,000-fold loss of infectivity.

Finally, attempts were made to recover virus after the introduction of HIV-contaminated blood into tap water. Venous blood obtained from two AIDS patients, representing whole-blood concentrations of 0.1, 0.5, 1.0, and 2.0% (vol/vol), was added to either PBS or dechlorinated tap water. No infectious HIV was detected as free virus in any sample tested as part of either experiment. A retrospective analysis of frozen plasma detected p24 antigen, at a level of about 30 pg/ml, in only one of the two samples. Thus, in one case, HIV was not detectable in the patient plasma, and in the second case, infectious titers were probably exceedingly low.

In contrast, infectious virus was recovered from PBLs introduced into tap water in this experimental series. For one blood sample (p24 plasma negative), only those samples containing 2% whole blood yielded HIV by culture and only at time zero in tap water (data not shown). In the second experiment, infectious virus was recovered from both 1 and 2% whole-blood samples (Table 1). HIV was cultured from PBS samples collected throughout the 2-h monitoring time. In comparison, virus was recovered for only 1 min (1% [vol/vol]) and 5 min (2% [vol/vol]) when blood was introduced into tap water.

In the same experimental series, DNA was recovered from constant sample volumes at the times indicated and subjected to PCR amplification of both HIV long terminal repeat and eukaryotic actin gene sequences. All of the samples contained amplifiable DNA on the basis of actin gene detection (data not shown). In the experiment detailed in Table 1, proviral DNA was detected when as little as 0.2% of the water volume was whole blood (i.e., 0.1 ml of blood in 50 ml of water; Fig. 4A), with 1% (0.5 ml) and 2% (1.0 ml) concentrations yielding proportionately more amplified DNA product. However, in spite of the concomitant loss of PBL viability and HIV infectivity, no diminution of PCR product was observed between time zero and 1 h (Fig. 4B)

![Figure 3. Recovery of infectious virus from HIV-infected PBLs. Pooled PBLs from HIV-negative donors were infected in vitro with HIV. When 10% of the cells expressed HIV p24, PBLs were added to the suspending medium at a concentration of 1 x 10⁶ to 2 x 10⁶ cells per ml, and both cell viability and the recovery of cell-associated HIV were monitored. (A) Cell viability based on trypan blue exclusion; (B) PBLs collected by centrifugation and assayed for HIV infectivity by coculture with susceptible cells as described in the text.](http://aem.asm.org/)
when identical volumes of water were sampled for DNA. By 2 h, the signal from amplified DNA specific for HIV proviral sequences was reduced by about 50% on the basis of densitometer analysis. Similar results were observed for sequences specific for the actin gene (data not shown).

**DISCUSSION**

Historically, the concern of environmental virologists has focused on viral agents which replicate to high numbers in the gastrointestinal tract and are spread by the fecal-oral route (e.g., hepatitis A and Norwalk viruses). The devastating outcomes associated with infection by HIV have led a few individuals to question the potential exposure of sanitary workers to this virus. To pose a realistic threat in this context, HIV must first enter and remain viable in the wastewater collection system. In this context, the first environmental exposure of blood-borne HIV would be to tap water.

Results from this study indicate that either free or cell-associated HIV added to dechlorinated tap water can lose 90% of its infectivity within 1 to 2 h and 99.9% within 8 h. When compared with poliovirus in parallel studies, this enveloped virus was decidedly labile. The presence of human serum in tap water slowed the loss of HIV infectivity through 8 h but did not stabilize virus through 24 h. Notably, the rate of HIV inactivation observed in this study is substantially faster than those previously published (3, 23).

Several differences exist between earlier studies and this report. Slade et al. (23) sterilized all of the water sources which they tested, including dechlorinated drinking water, settled raw sewage, and seawater. No further physical-chemical descriptions of these suspending media were given. However, their testing was conducted at the lower temperature of 16°C, which could account, in part, for the mean time of 1.8 days calculated to achieve a 10-fold reduction of HIV in tap water.

The study of Casson et al. (3) employed sterile distilled water, primary effluent with average total suspended solids of 230 mg/liter, and nonchlorinated secondary effluent with average total suspended solids of 14 mg/liter. At a temperature of 25°C, HIV infectivity was reduced 10-fold by 6 h in sterile water. A similar reduction in both primary and secondary effluents required 12 h. The somewhat enhanced survival of HIV in the presence of suspended solids and organic loads is not surprising, since this finding has long-standing precedence in work with enteric viruses in water systems.

Another methodological difference may exist in that efforts were made in this study to disperse HIV stocks before they were used in viral stability testing. The presence of aggregated virions could contribute to a finding of prolonged survival. Theoretically, an aggregate of 10 infectious particles could be measured as a single infectious unit until all 10 virions had been inactivated. Indeed, this logic may explain the somewhat-enhanced survival of HIV recovered from infected lymphocytes in tap water. A single cell replicating HIV, although no longer viable, would have numerous virions at the cell membrane and would require multiple inactivation events to render it noninfectious.

The efficacy of using PCR amplification for the detection of HIV was also evaluated as part of this study. Parallel tests using PCR amplification of reverse-transcribed cDNA to detect non-cell-associated HIV virion RNA after the introduction of contaminated blood into tap water were unsuccessful (data not shown). However, on the basis of p24 antigen capture analysis of plasma collected from corresponding whole-blood samples, it is not clear whether either sample contained intact HIV in sufficient quantity to have been recovered once the blood was diluted in water. Conversely, proviral DNA sequences present in infected lymphocytes were readily demonstrated even when as little as 0.1 ml of blood was added to 50 ml of water (1:500 dilution). However, there was no apparent correlation between the recovery of infectious HIV from lymphocytes and the detection of HIV proviral sequences. Both cellular DNA and viral DNA were detected in the absence of viable cells and infectious virus. Not until 2 h had elapsed was any diminution in amplified sequence observed. These results may be explained, in part, by the absence of significant levels of de novo proteases and nuclease in relatively clean water systems such as tap water.

Viruses such as HIV rely on an intact envelope for their in vivo infectivity. The virion envelope is a modified membrane derived from the host cell and containing virally encoded proteins responsible for receptor-mediated infection of target cells. Both the viral envelope and cellular membranes are susceptible to disruption under conditions of unfavorable osmotic pressure. Once the lipid bilayer surrounding the virus has been lysed, viral proteins required for infectivity are lost. Under these conditions, noninfectious HIV theoretically could still be detected as either virion capsid proteins...
References


