

Inactivation of Human Immunodeficiency Virus Type 1 by Ozone In Vitro

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A device was designed to deliver a constant source of given concentrations of ozone to fluids containing human immunodeficiency virus type 1 (HIV-1). Ozone was found to inactivate HIV-1 virions in a dose-dependent manner. Greater than 11 log inactivation was achieved within 2 hours at a concentration of 1,200 ppm ozone. Similar concentrations of ozone had minimal effect on factor VIII activity in both plasma and immunoaffinity-purified preparations of factor VIII treated

for the same time period. The data indicate that the antiviral effects of ozone include viral particle disruption, reverse transcriptase inactivation, and/or a perturbation of the ability of the virus to bind to its receptor on target cells. Ozone treatment offers promise as a means to inactivate human retroviruses in human body fluids and blood product preparations.

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HUMAN immunodeficiency virus type 1 (HIV-1), the etiologic agent of acquired immunodeficiency syndrome (AIDS), is a lentivirus that completes its replicative cycle by budding through the host cell membrane, acquiring host-derived and virus-encoded components in the process. Subsequent rounds of replication require an intact lipid envelope containing the virally encoded envelope proteins necessary for receptor binding.¹ It has been suggested that perturbation of the HIV-1 envelope may be a suitable approach to inactivating HIV-1.² Compounds that fluidize membranes by removing cholesterol (AL721)³ or by complexing membrane cholesterol (amphotericin B methyl ester)⁴ can inhibit HIV-1 replication in vitro. Although their precise mode of action has yet to be defined, these compounds may reduce HIV-1 infectivity by perturbing the envelope of HIV-1. We therefore investigated the activity of another membrane active agent, ozone, on HIV-1 infectivity in vitro.

Ozone, the triatomic allotrope of oxygen, is an extremely potent oxidant that has been shown to possess broad spectrum antimicrobial activity.⁵⁻⁸ It has been widely used in sewage treatment, in water purification, and in medicine.^{9,10} In particular, ozone has been shown to be effective against a number of enveloped and nonenveloped viral species, with enveloped viruses being more susceptible to ozone inactivation than those lacking lipid envelopes.^{5,11,12} We report on the in vitro inactivation of HIV-1 in cell culture media and deliberately infected factor VIII preparations at concentrations of ozone that are not toxic to target cells, while maintaining the biologic activity of factor VIII. We also

investigated the mechanism by which ozone mediates its antiviral effect.

MATERIALS AND METHODS

Cells and virus. The CD4a⁺ T-cell line HUT 78¹³ was maintained in RPMI 1640 + 10% heat-inactivated fetal bovine serum (FBS) + 1% penicillin/streptomycin (complete RPMI; all from GIBCO, Grand Island, NY) at 37°C, 5% CO₂ humidified atmosphere. HUT 78 cells served as the target cells in the HIV-1 inactivation studies. HUT 78/HIV-1_{AAV} is a cell line stably infected with an isolate of HIV-1 from our laboratory (AAV).¹⁴ HUT 78/HIV-1_{AAV} served as the source of HIV-1 for the inactivation studies. Cell-free conditioned media from HUT 78/HIV-1_{AAV} (HIV-1_{AAV} CM) was harvested by centrifugation (1,200g) and filtered through a 0.45- μ m filter (Nalgene, Rochester, NY). This virus preparation was titrated for production of progeny HIV-1 p24 in HUT 78 according to the infectivity assay described below.

Factor VIII. Factor VIII preparations used were either nonconcentrated, frozen, HIV-1-negative human plasma or immunoaffinity-purified human factor VIII (Monoclate; Armour Pharmaceuticals, Kankakee, IL). Factor VIII activity was determined in a one-stage assay.¹⁵

Ozone generation and delivery. Ozone was generated from medical grade oxygen by electrical corona arc discharge in a commercial ozone generator (Hansler GmbH, Iffezheim-Baden, Germany). Ozone was delivered into cell culture medium or factor VIII preparations through a closed hollow fiber system (Fig 1A) as a stream of ozone/oxygen in carrier nitrogen.¹⁶ The hollow fiber cartridge, virus reservoir, peristaltic pump, and all connecting lines were confined to a laminar flow biohazard hood. Figure 1B shows a closeup diagram of the hollow fiber gas exchange cartridge. The micropores of the hollow fiber are of sufficient size to allow for ozone to diffuse from the extracapillary space into the liquid phase, but small enough to prevent liquid from leaking back into the extracapillary space. Ozone and the liquid phase were pumped in a countercurrent fashion with respect to one another to maximize ozone diffusion. Ozone levels in the system were measured using a Dasibi model 1008 ozone monitor (Dasibi, Glendale, CA).

HIV-1 inactivation studies. For this and all studies described, 300 mL of Hanks' balanced salt solution (HBSS) were passed for 1 hour at a flow rate of 100 mL/min through the hollow fiber cartridge, reservoir, and connecting lines to moisten the fibers and other components. The system was then drained and 450 mL of cell-free HIV-1_{AAV} CM containing one-half of an infectious unit of HIV-1 was added to the reservoir. For our purposes, one-half of an infectious unit is defined as the dilution of virus that yields 50% of the maximum amount of progeny HIV-1 p24 antigen 5 days postinfection in the virus infectivity assay described below. The titration curve for virus used in these assays is shown in Fig 2. As can be seen, the detection of expressed HIV-1 p24 is linear up to a concentration of 1:4 dilution of input virus, with a maximum detection of 3,000 pg of HIV-1 p24. Hence, a dilution of approximately 1:24 of stock virus was used as one-half of an infectious unit.

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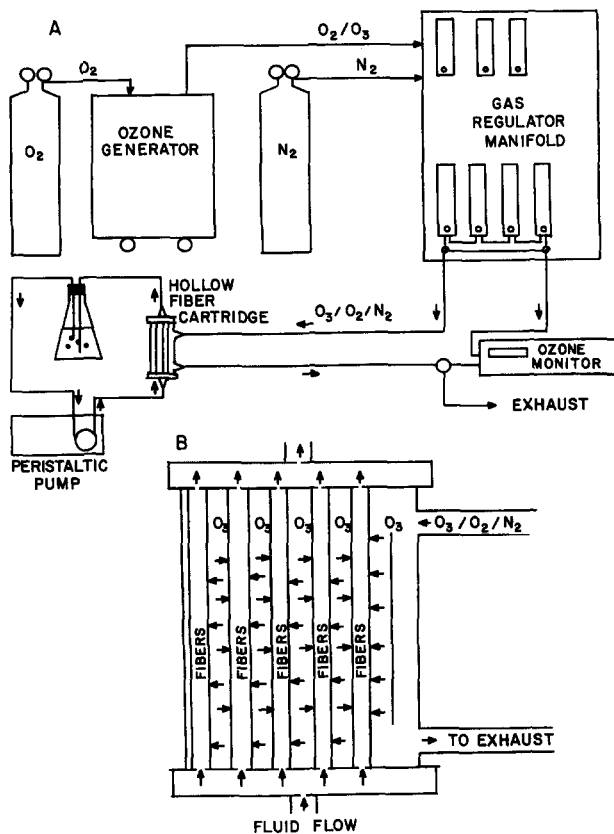


Fig 1. Schematic diagram of ozone generation/delivery system (A) and close-up of gas exchange cartridge (B) showing flow of oxygen, ozone and nitrogen gases, and HIV-1-containing fluids. Note that the input and exhaust ozone is monitored.

A full infectious unit would be a dilution of 1:12. The CM (final protein concentration, 35 mg/mL) was then pumped through the system at a rate of 100 mL/min. Ozone flowed through the extracapillary space of the hollow fiber cartridge at the concentrations and times indicated. To find a threshold virucidal dose of ozone, ozone concentrations were sequentially elevated on an hourly basis (ie, 1 hour at 200 ppm, 1 hour at 400 ppm, etc) (Figs 3A and 4A). The time parameter (abscissa) is a composite of the total time of exposure. For N₂ only experiments, carrier N₂ alone flowed through the cartridge. For pump only experiments, no gas was introduced into the cartridge, although the cartridge ports were opened to filtered room air. At the times indicated, 1 mL samples of treated CM were removed for use in the virus infectivity assay described below.

HIV-1 infectivity assay. One million HUT 78 cells were pelleted in 15-cm³ sterile tubes and resuspended in 50 μL of HIV-1-containing CM. Cells were exposed to the CM for 1 hour at 37°C and 5% CO₂ in wells of a 24-well plate. Five days postinfection the CM were sampled and assayed for viral p24 using a commercial antigen capture assay (Cellular Products, Buffalo, NY).¹

In those experiments investigating the degree to which ozone inactivates HIV-1, the HIV-1 infectivity assay was modified.¹⁷ Serial 10-fold dilutions of nonozonated HIV-1 were introduced into the assay system while ozone-treated HIV-1-spiked CM or human plasma was used undiluted. The cultures were assayed for HIV-1 p24 twice a week up until day 28. Results are expressed as a mean of a minimum of six separate experiments, each consisting of triplicate runs. Results are expressed as picograms per milliliter

HIV-1 p24 or percent untreated control. The cutoff for positive is 30 pg/mL of HIV-1 p24.

Ozone cytopathic effects. To determine if ozone is causing the formation of products in cell-free CM that are deleterious to the target cells used in the infectivity assays, sham infections were performed. First, cell-free HUT 78 cell culture-CM was filtered and added to the cartridge. It was then treated with either room air, N₂, or 1,200 ppm ozone for 2 hours. Mock infections were then performed by placing 10⁶ HUT 78 cells in 50 μL of the above treated CM versus control for 1 hour at 37°C. Cells were then washed and placed into culture at a concentration of 5 × 10⁵/mL. Four days later, cells were pulsed with 1 μCi [³H]-thymidine. Eighteen hours later, cells were harvested, washed, and precipitated with 10% ice-cold trichloroacetic acid for 2 hours on ice. Acid-precipitable material was collected on Whatman GF/C filters (Whatman Intl Ltd, Maidstone, England), washed, and counted. Results are expressed as counts per minute incorporated or a percentage of untreated control. Similarly, cells were assayed for viability via trypan blue dye exclusion.

To further evaluate any potential effects of ozone-treated media on the target cells, which would influence their ability to be infected by HIV-1, we analyzed the effect of the various treated CM on CD4a levels on the HUT 78 cells. HUT 78 cells were exposed for 1 hour at 37°C to media treated as above and were then stained with fluorescein isothiocyanate (FITC)-conjugated OKT4a (Ortho Diagnostics, Raritan, NJ) and analyzed on a Coulter EPICS V Flow Cytometer (Coulter Electronics, Hialeah, FL). Results were expressed as percent cells positive and mean channel number of fluorescence.

Effect of ozone on factor VIII levels and HIV-1 in factor VIII preparations. Frozen factor VIII containing human plasma prepa-

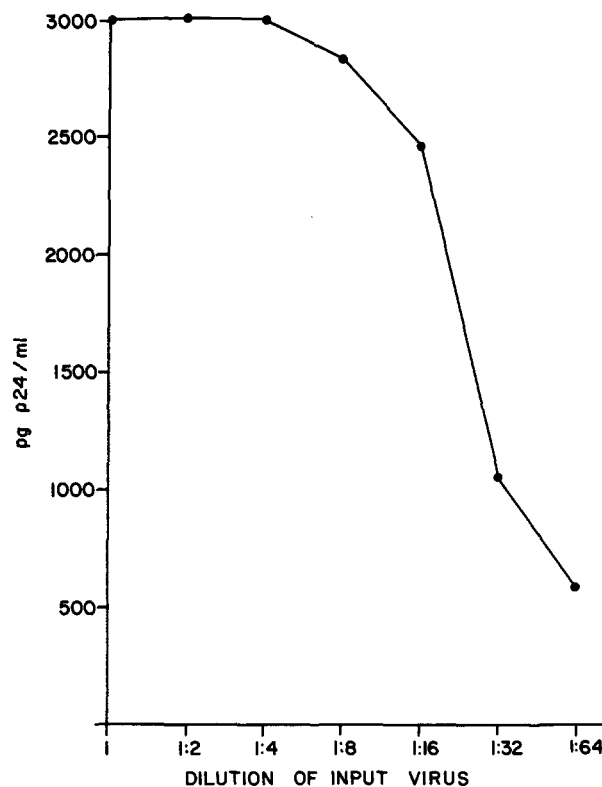


Fig 2. Titration curve of input virus in HIV-1 infectivity assay. Progeny HIV-1 p24 was measured 5 days postinfection by antigen capture assay.

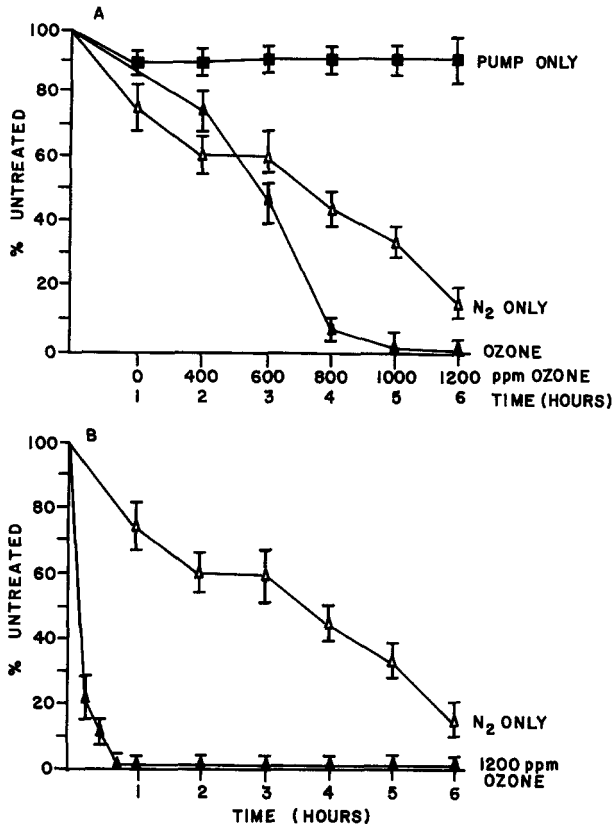


Fig 3. Effect of ozone on HIV-1 infectivity in cell culture medium using an escalating dose regimen (A) and continuous high-dose (1,200 ppm) (B) ozone. Virus infectivity was assayed after treatment with room air (■), nitrogen atmosphere (△), or ozone in carrier gas (▲). Residual infectious HIV-1 levels were assessed using the 5-day HIV-1 infectivity assay. CM from cultures 5 days postinfection were assayed for HIV-1 p24. Results are expressed as a percentage of the p24 produced by cultures infected with nonozonated HIV-1. The results shown are the mean values and ranges from three separate experiments. Each separate treatment (A: pump 1 to 6 hours, N₂ only 1 to 6 hours, escalating dose ozone; B: 1,200 ppm 1 to 6 hours) was tested in triplicate cultures within an individual experiment. Please note that the total concentration of ozone multiplied by time in (A) is 4,000 ppm hours and in (B) is 7,200 ppm hours.

rations were thawed at 4°C in an ice water bath and pooled. Pooled material was separated into two 250 mL aliquots. The first aliquot was diluted with 250 mL Dulbecco's phosphate-buffered saline (PBS) (pH 7.5) and added to the hollow fiber system (final protein concentration, 400 mg/mL). It should be noted that for this particular experiment the hollow fiber system was prewashed with Dulbecco's PBS for 1 hour before addition of diluted factor VIII. A sample of diluted factor VIII was removed and the remaining diluted factor VIII was pumped through the hollow fiber system. One hour, no gas (pump only) and one hour, nitrogen only (N₂ only) samples were removed. Ozone was then flowed through the system at the sequentially escalating concentrations indicated and samples were removed at hourly intervals. All samples were frozen for later assay of factor VIII activity.

The second 250 mL aliquot of factor VIII was combined with 250 mL of cell-free HUT 78/HIV-1_{AAV} containing a full infectious unit of HIV-1. This preparation (final concentration, 435 mg protein/mL) was then added to the hollow fiber system prewashed with HBSS. The HIV-1-containing factor VIII preparation was pumped

through the hollow fiber system and sequentially treated with room air only, N₂ only, and escalating ozone concentrations, as above. Samples were removed at the times indicated and 50 µL of this material was used to infect 1 × 10⁶ HUT 78 cells, as described above. CM were sampled 5 days postinfection and assayed for HIV-1 p24.

Next, a similarly prepared aliquot of human plasma diluted in PBS and spiked with HIV-1 (final protein concentration, 435 mg/mL) was treated in the hollow fiber system continuously at 1,200 ppm ozone over 6 hours. At various time intervals, samples were removed and analyzed for either HIV-1 infectivity or factor VIII activity.

Finally, 14 vials of affinity-purified factor VIII were reconstituted with 5 mL of H₂O each and diluted with 80 mL of PBS containing 1% human serum albumin. This mixture was added to 150 mL of HIV-1_{AAV} CM. After circulating this material several times through the moistened cartridge, the final protein concentration was 35 mg/mL (primarily from the HIV-1_{AAV} CM) and the final factor VIII concentration was 10 IU/mL. This material was then subjected to treatment with 1,200 ppm ozone for 6 hours and analyzed periodically for HIV-1 infectivity and factor VIII activity.

Mechanism of ozone inactivation of HIV-1. To ascertain whether ozone inactivated HIV-1 by directly perturbing the lipid envelope

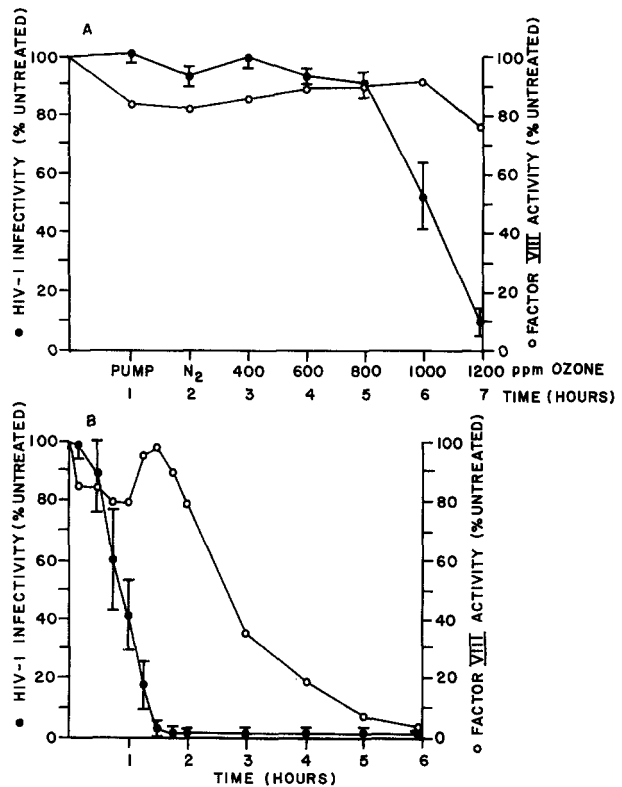


Fig 4. Effect of ozone on HIV-1 infectivity (●) and factor VIII activity (○) in factor VIII plasma using escalating dose (A) and continuous high-dose (1,200 ppm) (B) ozone. Residual infectious HIV-1 levels were assessed using the 5-day HIV-1 infectivity assay. CM from cultures 5 days postinfection were assayed for HIV-1 p24. Results are expressed as percentage of the p24 produced by cultures infected with nonozonated HIV-1. The values are taken from triplicate cultures and are expressed as mean values and ranges. Factor VIII activity was assessed by a one-stage assay and is an average of duplicate determinations. The ranges are so tight that they are not shown. Please note that the total concentration of ozone multiplied by time in (A) is 4,000 ppm hours and in (B) is 7,200 ppm hours.

of the virus, ozone-treated and nontreated HIV-1 preparations were analyzed for the presence of intact virions on sucrose density gradients.¹⁸ Briefly, untreated HIV-1 preparations or preparations treated continuously with 1,200 ppm ozone for 2 hours were concentrated by centrifugation at 40,000g for 2 hours and applied to a 22% to 65% continuous sucrose gradient. Samples were centrifuged at 30,000g for 12 hours. Fractions were collected and analyzed for HIV-1 p24 by antigen capture,¹ for reverse transcriptase,¹⁸ for HIV-1 gp120 and gp41 by Western blot analyses using human polyclonal anti-HIV-1 sera,¹ and for buoyant density by refractometry.¹⁸ Amounts of HIV-1 reverse transcriptase and p24 present as viral cores, intact virions, or soluble proteins were estimated by calculating the surface area of each entity between the fractions with buoyant densities of 1.26 to 1.21; 1.20 to 1.14; and less than 1.14 g/mL, respectively.

Effects of ozone on HIV-1 envelope protein cell surface receptor interactions were assessed in a competitive binding assay using rhodamine-labeled HIV-1 particles.¹⁹ Briefly, HIV-1 particles were banded on a sucrose gradient, concentrated on a glycerol cushion, and labeled with a rhodamine-linked saturated hydrocarbon, 18 carbons long (R-18) (Molecular Probes, Junction City, OR). Labeled particles were then separated from free R-18 by another centrifugation through 30% glycerol onto a 100% glycerol cushion. Labeled virions were then exposed to HUT 78 cells for 30 minutes at 4°C after the target cells had been pre-exposed to ozone-treated or nontreated, sucrose-banded HIV-1 virions, normalized for p24 and reverse transcriptase content. The degree of rhodamine-labeled HIV-1 binding to the target HUT 78 cells was analyzed on the Epics V flow cytometer.

RESULTS

Figure 3A shows the results of an escalating dose regimen on inactivation of cell-free HIV-1. With respect to ozone, the sample was treated sequentially for 1 hour at each of the indicated ozone concentrations. This escalating dose study was designed to define the minimum concentration of ozone at which significant (at least 2 log) virus inactivation was achieved. All curves represent the average of three separate experiments. As can be seen, there was a dose-dependent and time-dependent inactivation of cell-free HIV-1 associated with its passage through this hollow

fiber system. The relative contributions of room temperature incubation, mechanical shear, hypoxic atmosphere, and ozone to viral inactivation can be seen in Fig 3A. It is evident that, while ozone is perhaps the major contributing factor to the inactivation of HIV-1 in this system, it is by no means the sole mediator of this event. The results using this escalating dose regimen showed that a threshold dose of 1,200 ppm achieved ≥ 2 log inactivation of virus. The total concentration multiplied by time at this point was 4,000 ppm hours.

Figure 3B shows the results of the time-dependent inactivation of HIV-1 in cell culture medium using 1,200 ppm ozone. As can be seen, 1,200 ppm achieves ≥ 2 log virus inactivation within 45 minutes (900 ppm hours). Inactivation due to room temperature, mechanical shear, and nitrogen atmosphere is only approximately 25% within 1 hour. Hence, ozone is the major contributing factor to virus inactivation at both the higher dose and early time points.

Figure 4A shows the results of the effect of a sequentially escalating dose of ozone on HIV-1 viability and factor VIII biologic activity in an HIV-1-spiked human plasma preparation. As can be seen, there is minimal loss of factor VIII biologic activity associated with ozone treatment at concentrations that eventually inactivate HIV-1. Treatment to a threshold dose of 1,200 ppm results in a 90% inactivation of HIV-1 with only a corresponding 25% loss of factor VIII biologic activity.

Figure 4B shows the results of the time-dependent inactivation of HIV-1 and factor VIII biologic activity in a plasma preparation using continuous 1,200 ppm ozone. As can be seen, 1,200 ppm ozone achieves ≥ 2 log inactivation of HIV-1 by 2 hours while the corresponding factor VIII activity remains high (90%).

Similar results were seen when immunoaffinity-purified factor VIII preparations were inoculated with HIV-1 and treated continuously with 1,200 ppm ozone (Fig 5). In this instance, ≥ 2 log viral inactivation was achieved within 1

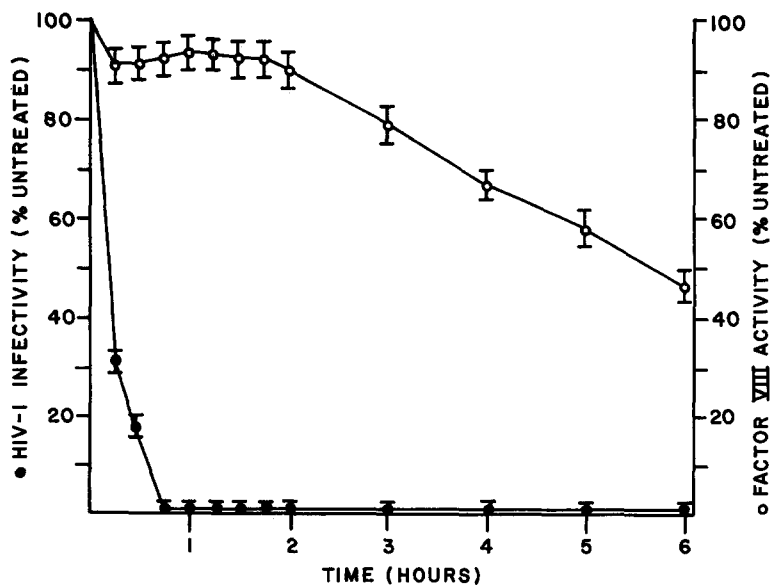


Fig 5. Effect of ozone on HIV-1 infectivity (●) and factor VIII activity (○) in immunoaffinity-purified factor VIII (monoclate) preparations using continuous high-dose (1,200 ppm) ozone. Residual infectious HIV-1 levels were assessed using the 5-day HIV-1 infectivity assay. CM from cultures 5 days postinfection were assayed for HIV-1 p24. Results are expressed as a percentage of the p24 produced by cultures infected with nonozonated HIV-1. The values presented are taken from triplicate cultures and are expressed as mean values and ranges. Factor VIII biologic activity was determined by one-stage assay and is the mean and ranges of triplicate determinations.

Table 1. Comparison of Ozone-Treated HIV-1 With a Titration of Untreated HIV-1 in a 28-Day HUT 78 Cell Culture System

HIV-1 Viral Input*	HIV-1 p24 Assay Result for Each Biweekly 28-Day Culture Sample (day in culture)†								
	1	4	7	10	14	17	21	24	28
3.5×10^1 ng HIV-1 in CM treated 2 h, 1,200 ppm ozone	-	-	-	-	-	-	-	-	-
3.5×10^1 ng HIV-1 in human plasma treated 2 h, 1,200 ppm ozone	-	-	-	-	-	-	-	-	-
3.5×10^1 ng HIV-1 no ozone	-	+	+	+	+	+	+	+	+
3.5×10^0 ng HIV-1 no ozone	-	+	+	+	+	+	+	+	+
3.5×10^{-1} ng HIV-1 no ozone	-	-	+	+	+	+	+	+	+
3.5×10^{-2} ng HIV-1 no ozone	-	-	+	+	+	+	+	+	+
3.5×10^{-3} ng HIV-1 no ozone	-	-	+	+	+	+	+	+	+
3.5×10^{-4} ng HIV-1 no ozone	-	-	+	+	+	+	+	+	+
3.5×10^{-5} ng HIV-1 no ozone	-	-	+	+	+	+	+	+	+
3.5×10^{-6} ng HIV-1 no ozone	-	-	+	+	+	+	+	+	+
3.5×10^{-7} ng HIV-1 no ozone	-	-	+	+	+	+	+	+	+
3.5×10^{-8} ng HIV-1 no ozone	-	-	-	+	+	+	+	+	+
3.5×10^{-9} ng HIV-1 no ozone	-	-	+	+	+	+	+	+	+
3.5×10^{-10} ng HIV-1 no ozone	-	-	-	-	-	+	+	-	+
3.5×10^{-11} ng HIV-1 no ozone	-	-	-	-	-	-	-	-	-
0 ng HIV-1 p24 no ozone	-	-	-	-	-	-	-	-	-

*Total HIV-1 viral input as measured by p24 antigen capture of 50 μ L of cell-free HUT 78 HIV-1_{AAV} cell culture CM either undiluted (3.5×10^1 ng) or serially diluted in virus-negative culture media.

†The mean of six separate experiments each run in triplicate being ≥ 30 pg/mL of HIV-1 p24. All 18 replicates of data points listed as negative were negative, and all replicates of data points listed as positive were positive save for day 24 of the 3.5×10^{-10} cultures, in which the mean was 28 pg/mL and some replicates were positive and some negative. A culture was deemed positive if two successive samples were positive with the second being either four times the first or ≥ 250 pg/mL of HIV-1 p24. All cultures save for the ozone-treated HIV-1, 0 pg HIV-1 p24, and 3.5×10^{-11} ng HIV-1 p24 were positive.

hour, whereas factor VIII activity after 2 hours was approximately 90%.

As can be ascertained from Figs 3, 4, and 5, increasing amounts of protein and possibly other plasma components decreased the virucidal effect of ozone. The HIV-1-spiked material in Figs 3 and 5 has a protein concentration of 35 mg/mL, while the human plasma in Fig 4 has a concentration of 400 (A) or 435 (B) mg/mL.

In an effort to determine the degree of inactivation of HIV-1 by ozone, HIV-1-spiked CM (35 mg protein/mL) and HIV-1-spiked plasma (435 mg protein/mL) were each treated with 1,200 ppm ozone for 2 hours. Fifty microliters of each of these treated materials (initially containing 35 ng of HIV-1 p24) were placed in a 28-day HIV-1 culture assay and compared with a serial dilution of untreated HIV-1 virus. In this experiment the transmission of an 11 log dilution of untreated virus could still be detected, whereas the transmission of ozonated, undiluted HIV-1 in CM or human plasma could not be discerned, suggesting that 1,200 ppm of ozone for 2 hours achieved a minimum of an 11 log inactivation (Table 1).

Studies were performed to evaluate the mechanisms of antiviral action of ozone treatment. There were no appreciable effects of ozone-treated CM on the viability, rates of DNA synthesis, and CD4a levels of HUT 78 target cells exposed in mock infections (Table 2). However, treatment of HIV-1-infected CM with 1,200 ppm ozone did result in a marked decrease in recoverable sucrose-banded virions as measured by reverse transcriptase and HIV-1 p24 analyses (Fig 6). In six separate experiments comparing the surface area under the curve for reverse transcriptase activity and p24 antigen content between media treated and untreated with ozone, the loss of recoverable virions ranged from 75% to 93% with a mean of 85%. Western blot analysis of the fractions in Fig 6 for HIV-1 gp41 and gp120 envelope protein content showed a loss of antigen comparable with that seen with reverse transcriptase and p24 gag protein (data not shown). Hence, there was no preferential loss in residual intact virions of envelope antigen or functional reverse transcriptase activity secondary to ozone.

As shown in Fig 6, there was no appreciable accumulation of viral cores in the ozone-treated CM. All of the

Table 2. Effects of Ozone-Treated CM on HUT 78 Cells in a Mock Infection

Condition of CM	CD4a Levels		Cytopathic Effect			
	% Cells Positive	\bar{x} Channel No.*	Cell Viability ($\times 10^6$)	% Control	[3 H] Thymidine Incorporation (cpm)	% Control
No treatment	57 \pm 2	84 \pm 2	2.89 \pm 0.33	100	12,850 \pm 1,340	100
Room air (2 h)	59 \pm 2	77 \pm 2	2.73 \pm 0.24	94	10,280 \pm 985	80
N ₂ (2 h)	58 \pm 2	86 \pm 2	2.64 \pm 0.35	91	10,993 \pm 1,002	85
1,200 ppm ozone (2 h)	61 \pm 2	89 \pm 2	2.73 \pm 0.18	94	9,124 \pm 878	71

All assays were run six times and results equal mean \pm 3 SD.

*Channels are grouped from 1 to 267 and are on a log scale.

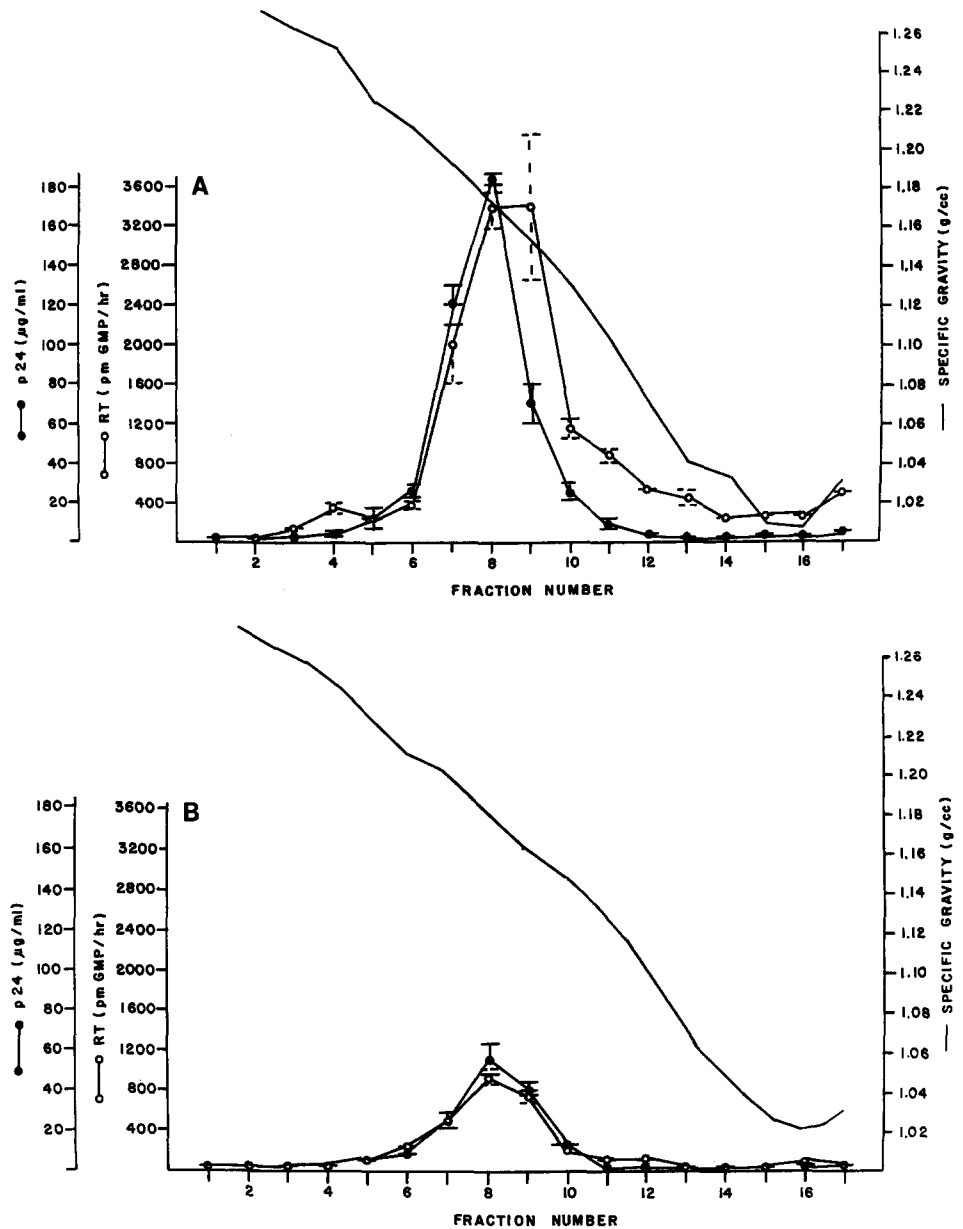


Fig 6. Analysis of fractions from sucrose density gradients of HIV-1-positive cell-free CM untreated (A) or treated with 1,200 ppm ozone (B) for 2 hours. Specific gravity, HIV-p24 (●--●), and reverse transcriptase (○--○) levels are shown. Specific gravity was determined by refractive index. HIV-1 p24 was determined by HIV-1 p24 antigen capture immunoassay and is expressed as micrograms per milliliter with mean values and ranges of triplicate determinations shown. Reverse transcriptase activity was determined using the template: primer, poly rC:oligo dG, 10 mM Mg²⁺, and [³H]-GTP. Results are expressed as picomoles per liter of GMP incorporated per hour and are the mean values and ranges from triplicate samples.

missing HIV-1 p24 antigen, but not the reverse transcriptase activity, was recovered in the supernatant after the original high speed pelleting step before sucrose banding (data not shown). This result would indicate that ozone treatment led to complete solubilization of most of the treated HIV-1 particles and that, while key epitopes of solubilized HIV-1 p24 could still be detected in the antigen capture assay, functional reverse transcriptase activity was lost secondary to the oxidation of solubilized enzyme.

Further evaluation of ozone-treated and untreated HIV-1 sucrose-banded virions for their ability to compete against rhodamine-labeled HIV-1 for cell surface receptor binding has shown an impaired ability of ozone-treated virions to displace HIV-1 (Table 3). This experiment was performed twice with similar results. Although further investigation is necessary, the implication is that ozone-treated virions have

a diminished capacity to bind to their natural cell surface receptor, CD4.

DISCUSSION

Reducing the infectious risk of human blood products is an area of intense research.²⁰⁻²² Inactivation of infectious agents in blood products under conditions that retain their intrinsic protein biologic activities has undergone extensive study. Thermal inactivation of viruses in blood derivatives has been marginally successful, due in part to the thermostability of these components.²³⁻²⁵ Methods ranging from gamma-irradiation,²⁶ porous membrane filtration,²⁷ and solvent/detergent mixtures²⁸⁻³¹ have been investigated. To date, tri(n-butyl)phosphate (TNBP) detergent mixtures have demonstrated a minimum 4 to 5 log 10 reduction in a

Table 3. Effect of Ozone on the Binding of HIV-1 Virions as Measured in a Competition Assay

Condition	Rhodamine-Labeled HIV-1	\bar{x} Channel No.*	\bar{x} Channel No. Minus Autofluorescence of HUT 78 Cells	% of Control*
HUT 78 cells	No	81	0	0
HUT 78 cells	Yes	155	74	100
HUT 78 cells preincubated with unlabeled HIV-1 not treated with ozone	Yes	122	41	34
HUT 78 cells preincubated with unlabeled HIV-1 treated with ozone	Yes	143	62	67

*Channels are grouped from 1 to 256 and are on a log scale. Calculation of percentage of control is performed by first converting \bar{x} channel number to an arithmetic value, subtracting autofluorescence of HUT 78 cells, and dividing by the value obtained with HUT 78 cells plus rhodamine-labeled HIV-1.

variety of lipid-enveloped viruses, including HIV-1, vesicular stomatitis virus, and Sindbis virus, with a concomitant high recovery rate of protein biologic activity. Analogous work in chimpanzees with TNBP-treated, exogenously viral-spiked blood samples with hepatitis B and non-A, non-B (Hutchinson strain) viruses has shown similar log reductions. However, methods eclipsing a 6 log viral reduction with a similar protein recovery are still being explored. In addition, methods that obviate the necessity for removal of the inactivating agent (ie, Sephadex G-25) and/or that are compatible with cellular systems would ultimately be preferable.

Ozone has previously been shown to possess potent antiviral activity, especially when used against lipid-enveloped viruses. For this reason we investigated the use of ozone as a potential anti-HIV-1 agent. We used a hollow fiber delivery system that maximizes the surface area available for ozone to interact with the fluid material of interest. The system also allows for the precise regulation of concentrations of ozone to be delivered into the hollow fiber cartridge. Afferent as well as efferent concentrations of ozone can also be monitored to determine if saturating levels of ozone are achieved in the treated material. With regard to laboratory safety concerns, this closed system has proven to be safe and leak-proof, thereby reducing possible exposure of laboratory personnel to ozone and human retroviruses to an absolute minimum.

We first examined the ability of ozone to inactivate cell-free HIV-1 in cell-free CM. These results indicate that ozone has potent anti-HIV-1 activity. Preliminary experiments using an escalating dose regimen indicated that a 1,200 ppm dose of ozone achieved a ≥ 2 log inactivation of virus. The data show that neither incubation of the virus preparation at room temperature and at atmospheric conditions for the duration of the experiment nor mechanical

shear created by pumping the virus through the system inactivate the virus to any great extent. However, there was a significant inactivation of the virus due to exposure of virus-containing CM to the stream of carrier nitrogen. Exposure of the virus to pure nitrogen for 6 hours results in an almost 85% inactivation of virus.

During exposure to nitrogen for 6 hours, conditions are completely anoxic and a maximal pH of 8.1 is achieved, whereas with exposure to room air the oxygen concentration is 21% and the maximal pH is 7.58. It is possible that anoxic conditions or pH changes seen with 6 hours of nitrogen exposure contribute to HIV-1 inactivation. Because some HIV-1 inactivation was observed with N_2 alone, it was necessary to determine the proportion of virus inactivation attributable to ozone over the course of a 6-hour treatment. Comparison of the time-dependent inactivation of virus seen with 1,200 ppm ozone with that seen with pure nitrogen clearly indicates that ozone plays the predominant role in achieving virus inactivation for periods up to 6 hours. Ozone inactivation of HIV-1 occurs relatively quickly compared with any inactivation observed with N_2 alone. Inactivation of HIV-1 at 45 minutes is ≥ 2 log when 1,200 ppm ozone is used and only 25% when nitrogen alone is administered.

Investigation of the degree of inactivation of HIV-1 in CM or human plasma by 1,200 ppm ozone for 2 hours indicates a minimum 11 log reduction in viral infectivity, as determined by a 28-day culture assay. Therefore, the effects of temperature, N_2 , and mechanical shear, which yield an approximate 0.22 log reduction in HIV-1 infectivity, are certainly eclipsed by the 11 log reduction in infectivity achieved by ozone.

We examined the ability of ozone to render blood products, such as factor VIII plasma preparations, free of HIV-1 without seriously damaging their biologic activity. The escalating dose study indicated that, at 1,200 ppm, ozone can achieve a 90% inactivation of HIV-1 in human plasma with only a corresponding 25% decrease in factor VIII biologic activity. Measurement of exhaust ozone levels indicated that saturating levels of ozone in the factor VIII preparations were not achieved in this experiment (data not shown) and, thus, longer exposures of HIV-1-containing factor VIII preparations at the higher ozone concentrations were used to eliminate the virus entirely. Use of 1,200 ppm ozone for a period of 2 hours achieved saturation and resulted in an 11 log inactivation of HIV-1 in the human plasma preparation. Under the same conditions, factor VIII biologic activity was diminished only 10%. Similarly, ≥ 2 log HIV-1 inactivation in immunoaffinity-purified factor VIII preparations occurred within 1 hour of exposure to 1,200 ppm of ozone with an approximately 10% loss of factor VIII.

Comparison of the results of inactivation of HIV-1 by high-dose ozone (1,200 ppm) in cell culture media, monoclone, and factor VIII plasma preparations indicated that a longer exposure time was necessary to achieve total virus inactivation in the plasma factor VIII preparation. The reason for the apparent protection of HIV-1 from the

effects of ozone in this experiment is most likely the increased protein levels in the factor VIII preparation. Such increased protein levels and other plasma components may act to protect the virus from the effects of ozone.³²

The exact mechanism by which ozone mediates the inactivation of HIV-1 remains to be elucidated. Our preliminary data indicate that, at the concentrations tested, ozone results in the solubilization of virions. However, the reduction in infectious virus (about 10¹¹) was far greater than the observed reduction in detectable virions (about 85%). This finding suggested that, in addition to the physical destruction of HIV-1 virions, there was a far greater functional impairment of these virions in the infectivity assay. Our data do not suggest that there was any obvious effect of ozone-treated CM on the viability and CD4a levels of the HUT 78 target cells used in the assay. Nor do our results indicate any preferential quantitative effect on HIV-1 envelope proteins and reverse transcriptase levels in the virions. However, there was some suggestion of a deleterious effect on virus binding to cell surface receptors.

Ozone may react with the unsaturated fatty acids in the

lipid envelope of the virus, thereby increasing membrane fluidity and resulting in viral inactivation. Alternatively, ozone may react with other components in the virus preparation (serum proteins, fatty acids, etc) to produce secondary reaction products, such as hydroxyperoxides, that in turn mediate the actual viral inactivation. Far more extensive analyses of all components of the HIV-1 life cycle will be required to fully elucidate ozone's antiretroviral activity.

Ozone has potent anti-HIV-1 activity in cell culture media and factor VIII preparations. Although the exact mechanism by which ozone mediates its effect remains unclear at this time, it is readily apparent that ozone may be of use in rendering factor VIII and possibly other blood products, both proteinaceous and cellular, free of HIV-1 and other infectious agents.

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