X-Irradiation Enhances *In Vitro* Human Immunodeficiency Virus Replication Correlation with Cellular Levels of cAMP

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Abstract. **Total body x-irradiation has been utilized in the treatment of several human diseases, including leukemia, where it is followed by bone marrow transplantation, and in some autoimmune disorders. Recently, it was reported that total body irradiation appeared useful in the treatment of Friend leukemia virus infection in mice. In this report, the effect of x-irradiation on the replication of human immunodeficiency virus (HIV) *in vitro* in CD4$^+$ cells was examined. MT-4 cells and HIV strain human T cell lymphotropic virus Type III$_B$ were used to conduct this study. Infected MT-4 cells were irradiated at the time of infection or following infection with x-ray doses of 25–300 cGy. Doses of 50, 150, and 300 cGy enhanced HIV replication by 1.6-, 2-, and 4.8-fold, respectively. Irradiating the cells prior to infection also resulted in similar enhancement of HIV replication. This phenomenon was also observed with wild-type HIV isolates grown in peripheral blood mononuclear and in HIV chronically infected cells. In addition, the enhancement was associated with a radiation-induced increase in intracellular levels of cAMP. The use of the cAMP-dependent protein kinase A inhibitor, H-8, inhibited HIV replication by 65%. These data suggest that *in vitro* exposure to low doses of x-ray enhances HIV replication partially via a cAMP-dependent pathway.**

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Treatment of leukemia by chemotherapy and total body irradiation (TBI) followed by bone marrow transplantation has long been established. Recently, it was reported that low dose TBI acted as a potent antiretroviral agent *in vivo* against Friend leukemia virus (FV) in mice (1). Thus, it was suggested that TBI might be useful in the treatment of acquired immunedeficiency syndrome (AIDS). AIDS is caused by human immunodeficiency virus (HIV), which, like FV, is a retrovirus. Infection with HIV is associated with a progressive deterioration of the patient's immune status due to a characteristic depletion of CD4$^+$ cells (2, 3). Patients with HIV infection frequently have experienced a long incubation period (5–10 years) before they develop frank AIDS. In contrast to FV, HIV does not cause leukemia; however, the incidence of malignancies, such as primary brain lymphoma, disseminated non-Hodgkins lymphoma, and Kaposi's sarcoma, among HIV-infected patients is higher than in the general population.

Zidovudine, the approved drug for treatment of HIV infection appears to decrease the incidence of opportunistic infections and prolongs the life span of patients with AIDS (4). Although it improves the quality of life for many patients and briefly improves their CD4 counts, it is not without its problems, which include bone marrow toxicity (5–7) and the recently reported emergence of zidovudine resistant HIV strains (8). The devastating effects of HIV infection and AIDS together with the limitations of the existing anti-HIV therapies warrant the search for new methods of therapy.

In light of the reported antiretroviral effects of TBI

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against FV, the effects of x-irradiation on HIV replication in an *in vitro* system utilizing $CD4^+$ cells were studied.

Materials and Methods

Cells and Virus. The MT-4 cell line used in conducting this study is a $CD4^+$ immortalized T cell line that was kindly provided by Dr. Blaine Hollinger of Baylor College of Medicine, Houston, TX. Cells were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 2 m*M* L-glutamine, 100 units/ml of penicillin, 100 μg/ml of streptomycin, 0.075% $NaHCO_3$, and 5 m*M* HEPES, and were maintained at 37°C.

Peripheral blood mononuclear cells (PBMC) were separated from heparinized blood obtained from healthy normal volunteers by standard Ficoll-Hypaque technique (9). Cells were stimulated with phytohemagglutinin for 72 hr in RPMI 1640 (with the same ingredients as described for MT-4 cells) and then used for experiments.

HIV strain human T cell lymphotropic virus Type III_B ($HTLVIII_B$) was used in these experiments unless otherwise mentioned. Virus stocks were prepared from H9 HIV chronically infected cells and titrated as described previously (10).

Wild-type HIV patient isolates were isolated by co-culturing 5×10^6 PBMC from HIV-infected patients with 5×10^6 of phytohemagglutin-activated PBMC from normal donors for 2 to 4 weeks in the presence of 10% recombinant interleukin 2. The phytohemagglutinin-activated normal PBMC (2×10^6) were added once a week to the co-cultures, and media was changed every 3–4 days. Reverse transcriptase activity of co-culture supernatants were determined once a week. Virus stocks were then prepared and titrated as described elsewhere (10).

Infection Experiments Using Cell-Free Virus. The MT-4, U937, or peripheral blood mononuclear cells (5×10^6) were infected with HIV at a multiplicity of 0.02–0.05 50% tissue culture infectious dose ($TCID_{50}$)/cell in 1 ml for 1 hr. The virus was then washed off and the cells were cultured at a density of 0.5×10^6/ml in RPMI medium, as mentioned above. Cell viability was determined by trypan blue exclusion.

Cell Irradiation. X-irradiation was performed with a Mark I ^{137}Cs irradiator model 68 (J. L. Shepard and Associates) at a dose rate of 7.68 Gy/min. The cells were irradiated in 0.5 ml of culture medium and then the original medium was added back.

Reverse Transcriptase Assay. Two ml of cell-free culture supernatants, collected at the time of harvest as indicated, were precipitated overnight at 4°C with 10% polyethylene glycol and 0.13 *M* NaCl. The reverse transcriptase (RT) activity of samples was then assayed in duplicate by adding 10 μl of each sample to 90 μl of a buffer made of 42 m*M* Tris-HCl (pH 7.8), 8.5 m*M* dithiothreitol, 10 m*M* $MgCl_2$, 3.4 m*M* NaCl, and 25 μCi/ml of [^{3}H]TTP (10–20 Ci/mmol; New England Nuclear Corp., Boston, MA) and 0.5 units/ml of oligo dT poly(rA) template primer (Pharmacia/P-L Biochemicals, Piscataway, NJ), as described previously (10).

cAMP Determination. Infected or uninfected cells (5×10^6) were pelleted at selected time intervals with low speed centrifugation (10 min at 1,500 rpm). The pellet was then resuspended in 1 ml of acidified ethanol until dry. The dry pellet was then resuspended in 0.4 ml of distilled H_2O and frozen at 20°C until assayed for cyclic nucleotide content (11). The cAMP was measured by radioimmunoassay using Amersham Kits (Arlington Heights, IL). Briefly (cAMP), 50 μl of unknown sample or standard were incubated with 100 μl of a cAMP-specific binding protein and 50 μl of [^{3}H]cAMP for 2 hr at 4°C. The free unbound nucleotide was then precipitated by adding 100 μl of a charcoal suspension to each tube and centrifuging for 2 min at 12,000 rpm in a high speed centrifuge. Two hundred microliters of the supernatants were then carefully removed and replaced in scintillation vials together with 10 ml of Aquasol cocktail (Dupont). The amount of unlabeled cAMP present was inversely related to the amount of labeled cAMP protein complex. The concentration of cAMP was then determined by comparison with a linear standard curve.

Results

Effect of X-Irradiation on HIV Replication. Since it seemed possible that TBI might be beneficial in the treatment of HIV infection, it was then of interest to know how radiation would affect HIV replication *in vitro*. In the first series of experiments, MT-4 cells were infected with HIV (strain $HTLVIII_B$) at a multiplicity of infection of 0.05 $TCID_{50}$/cell for 2 hr, then exposed to an x-ray dose ranging from 25 to 300 cGy (Fig. 1). At Day 4 after infection, supernatants were collected and tested for RT activity as a marker for HIV replication. The replication of HIV was enhanced by x-ray doses of 50–300 cGy, and the extent of the enhancement appeared to be dose dependent. Replication was enhanced by 1.6-, 1.8-, and 4-fold using 50, 150, and 300 cGy, respectively, when compared with HIV unirradiated infected cells. At the doses tested above, the susceptibility of the infected cells to the lethal effects of x-irradiation was not significantly different from that of uninfected cells (data not shown). To determine whether the effects on HIV replication were a result of a direct effect on HIV genome or indirectly through radiation-induced cellular mechanisms, uninfected MT-4 cells were exposed to increasing dosages of radiation and subsequently infected as described previously. The RT activity at Day 4 was again enhanced in a dose-

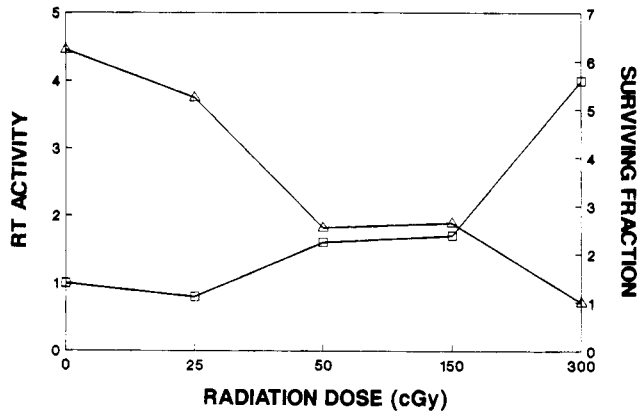

Figure 1. Effect of multiple doses of x-ray on HIV replication. MT-4 cells were first infected, then exposed to increasing doses of x-ray. HIV replication was then evaluated at Day 4 by determining RT activity that was expressed as cpm/ml/min ($\times 10^6$ (□), and the cell surviving fraction (△) was determined by trypan blue exclusion. The data presented are representative of three independent experiments.

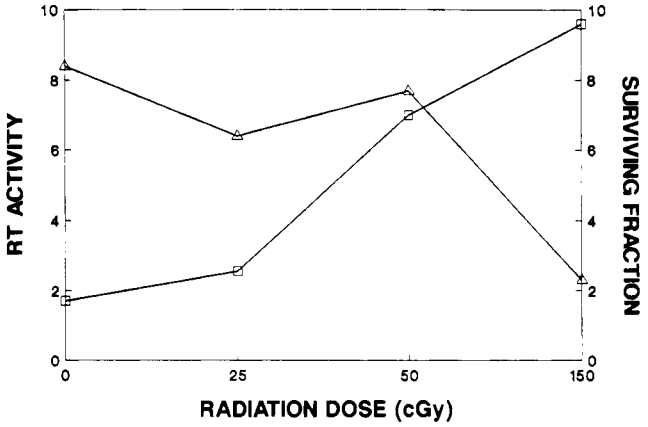

Figure 2. Effect of multiple doses of x-ray on HIV replication. Experimental protocol is the same as described in legend of Figure 1, except that the cells were irradiated first, then infected with HIV. RT activity (□) was expressed as cpm/ml/mn ($\times 10^6$) and surviving fraction of MT-4 cells (△) $\times 10^5$.

dependent manner. Replication of HIV was enhanced by 1.3-, 3.9-, and 5.3-fold using 25, 50, and 150 cGy as compared with infected unirradiated cells (Fig. 2). These data suggest that the enhancement of HIV replication was mediated by radiation-induced cellular enzymes.

To further confirm our observation, a time course experiment of the enhancement of HIV replication by x-irradiation was performed. Infected cells were exposed to 50 cGy and HIV replication was assessed every 24 hr through 96 hr. This dose was chosen because it was the lowest dose of irradiation that constantly produced a significant amount of enhancement. Figure 3 shows that HIV replication was enhanced at every time point examined from Day 1 through Day 4 after infection, as determined by RT activity of culture supernatants.

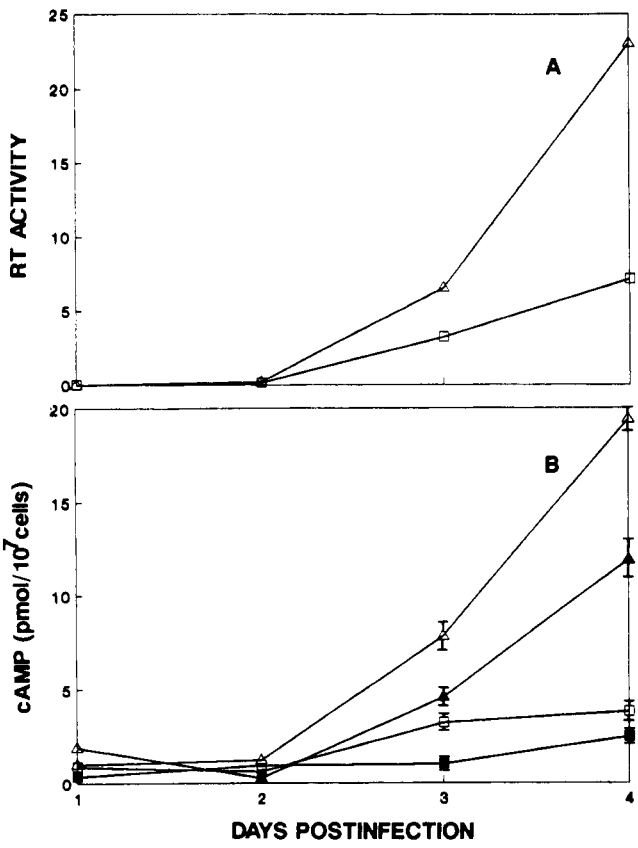

Figure 3. Time course for the effect of x-ray on HIV replication and cellular levels of cAMP. MT-4 cells were infected then x-irradiated with a dose of 50 cGy. (A) Then culture supernatants were tested daily for RT activity, unirradiated cells (□) and irradiated cells (△), and (B) cells were evaluated for their cAMP levels, unirradiated MT-4 (■), unirradiated HIV-infected MT-4 cells (▲), irradiated MT-4 cells (□), and HIV-infected irradiated MT-4 cells (△).

Effect of X-Irradiation on the Replication of HIV Fresh Patient Isolates. To determine whether the effects of the radiation-induced enhancement of HIV replication was conserved among HIV strains other than the laboratory adapted HIV 3b strain, the effects of 50 cGy of x-ray on the replication of three HIV fresh patient isolates were examined. Since the replication cycle of patient isolates tends to be slower than that of the laboratory strains, the infection was allowed to proceed to Day 4 after infection, before the cells were irradiated. The viral replication was then examined on Days 7, 11, and 14 after infection. As shown in Table I, 50 cGy of x-ray enhanced HIV replication by 4.1-, 5.5-, and 30-fold for patient strains HIV_{10D}, HIV_{10W}, and HIV_{10K}, respectively. Moreover RT activity in the x-irradiated patient isolate-infected cell cultures was detected 3 days earlier than in unirradiated cells. This suggests that the radiation-induced effects on HIV replication were not unique to HIV 3b strain, but were induced in wild-type HIV strains.

Effect of X-Irradiation on HIV Replication in PBMC. The effect of x-irradiation on HIV replication was next examined utilizing PBMC. The x-irradiation

Table I. Effect of X-Irradiation on the Replication of HIV Fresh Patient Isolates

Sample	Days post-infection	RT activity (cpm/ml/min) Nonirradiated	X-irradiated[a]	Fold increase
$HTLVIII_B$	7	9.0×10^5	1.7×10^6	1.9
	11	6.1×10^4	1.4×10^5	2.3
	14	3.4×10^4	1.4×10^5	4.0
HIV_{10D}	7	ND[b]	ND	
	11	ND	8.0×10^5	
	14	3.7×10	1.5×10^6	4.1
HIV_{10W}	7	ND	ND	
	11	ND	2.8×10^4	
	14	1.7×10^5	9.4×10^5	5.5
HIV_{12K}	7	ND	ND	
	11	ND	5.4×10^4	
	14	2.1×10^4	6.3×10^5	30

[a] MT_4 cells were infected with equal amounts of infectious units with different HIV strains, then irradiated on Day 4 after infection with 50 cGy of x-ray. The RT was then determined at the interval indicated.
[b] ND, not detectable.

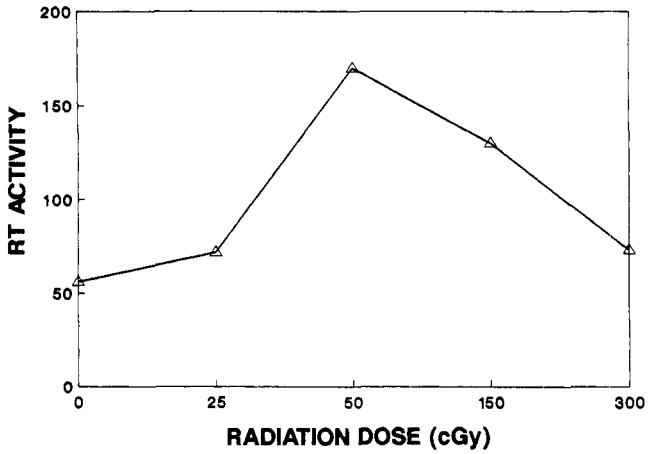

Figure 4. Dose response for the effect of x-irradiation on HIV replication in PBMC. PBMC were obtained from healthy HIV seronegative blood donors. Cells were infected with $HTLVIII_B$ then irradiated with increasing doses of x-ray. RT activity was determined at Day 4 after infection.

repeatedly enhanced the replication of HIV-3b strain by 2–3 fold at Day 7 after infection. Figure 4 shows the dose response for the effect of x-irradiation (25–300 cGy) on HIV replication. The RT activity increased by 1.3–3-fold depending on the dose. This level of enhancement was less than that observed in MT-4 cells, probably due to the heterogeneity of the PBMC.

The ability of x-irradiation to enhance the HIV replication of fresh patient isolates in PBMC was also examined. Table II shows that 50 cGy produced approximately a 2-fold increase in RT activity of the laboratory strain $HTLVIII_B$ as well as the wild-type isolates HIV_{18P} and HIV_{10C} at Days 7 and 11 after infection. This suggests that the observed enhancement of radiation-induced HIV replication was not unique

Table II. Effect of X-Irradiation on HIV Replication in PBMCs

Sample	Days post-infection	RT activity (cpm/ml/min) Nonirradiated	X-irradiated[a]	Fold increase
$HTLVIII_B$	7	7.4×10^4	1.7×10^5	2.3
	11	1.8×10^5	3×10^5	1.7
HIV_{18P}	7	2.4×10^5	5.3×10^5	2.2
	11	1.4×10^5	3×10^5	2.1
HIV_{10C}	7	3.4×10^5	5.7×10^5	1.7
	11	1.3×10^5	2.9×10^5	2.2

[a] Cells were infected with equal amounts of infectious HIV units, then irradiated at Day 4 after infection with 50 cGy of x-ray.

to the MT-4 cells, but was also produced in freshly isolated PBMC as well.

Effect of X-Irradiation on Cellular Levels of cAMP. To determine the possible cellular mechanisms that might be involved in the radiation-induced enhancement of HIV replication, the following experiments were restored. Previously, it was reported that exposure to x-irradiation induced an increase in tissue levels of cAMP (12–14). Because it is now known that the cAMP-dependent protein kinase A is involved in the regulation of cell activation (15) and recent reports from this laboratory suggest the involvement of cAMP in the replication of HIV (10, 16), the possible role of cAMP in the x-ray-induced enhancement of HIV replication was studied. Figure 3B shows the patterns of intracellular concentrations of the second messenger cAMP in HIV-infected MT-4 cells after exposure to 50 cGy of x-ray. A significant increase in intracellular levels of cAMP was observed in unirradiated HIV-infected cells as compared with uninfected controls by Day 3 after treatment and continued through Day 4 (Fig. 3B) that paralleled a similar increase in RT activity (Fig. 3A) in culture supernatants. This extended and confirmed the earlier observations (10) concerning the relationship between HIV replication and cellular levels of cAMP. Moreover, x-irradiation enhanced cellular levels of cAMP both in uninfected and HIV-infected cells. The enhancement in HIV replication by x-irradiation could be correlated with an increase in cellular levels of cAMP that occurred with the observed enhancement in HIV replication.

Effect of H-8 HIV Replication. To verify the relationship between cAMP-dependent protein kinase A and HIV replication, an inhibitor of protein kinase A, H-8 (17), was tested for its effect on HIV replication. In Table III it is shown that a dose of 1–10 μM inhibited HIV replication by about 65% at Day 4 after infection. These data indicate that HIV replication appears to be regulated, at least in part, by cAMP-dependent protein kinase A.

To further substantiate the role of protein kinase

Table III. Effect of H8 on HIV Replication[a]

HIV[b]	H8[c] (μM)	RT activity[d] (cpm/ml/min)	Percentage of inhibition
+	—	1.7×10^5	
+	0.1	1.1×10^5	34.5
+	1.0	5.7×10^4	67.6
+	10.0	6.3×10^4	64.0

[a] A representative of four independent experiments.
[b] MT-4 cells were infected with 0.05 $TCID_{50}$/cell with strain $HTLVIII_B$.
[c] Cellular cytotoxicity at the highest dose was 25%.
[d] Determined at Day 4 after infection.

Table IV. Effect of H-8 on the X-Irradiation-Induced Enhancement of HIV Replication[a]

H8[b]	X-irradiation[c]	RT activity[d] (cpm/ml/min)	Fold enhancement of HIV replication
−	−	4.3×10^4	
+	−	1.9×10^4	0.4
−	+	2×10^6	46.5
+	+	1.1×10^6	25.6

[a] A representative of two independent experiments, MT-4 cells were infected with 0.05 $TCID_{50}$/cell with strain $HTLVIII_B$.
[b] 10 μM.
[c] 50 cGy.
[d] Determined at Day 4 after infection.

A in the radiation-induced enhancement of HIV replication, HIV-infected cells, x-irradiated or nonirradiated, were treated with 10 μM of H-8, and the replication of HIV was determined at Day 4. As shown in Table IV, H-8 reduced the fold enhancement of HIV replication induced by x-irradiation from 46.5-fold to 25.6-fold.

Discussion

The data from these studies suggest that exposure of HIV-infected cells to x-irradiation enhanced their ability to replicate HIV. The enhancement was observed as early as 24 hr after infection with the $HTLVIII_B$ virus strain. The phenomenon was also observed with fresh patient virus isolates exposed to radiation, was dose dependent on the amount of exposure, and was not unique to MT-4 cells. Exposure of cells infected with fresh patient isolates to radiation not only increased HIV yield, but also shortened the eclipse period, because RT activity was detected a day earlier in the culture supernatants. It did not matter whether the cells were irradiated before or after the infection, indicating that the effect of radiation on viral propagation was through cellular events affected by the radiation, rather than through a direct effect on the virus itself.

The mechanism by which x-ray affected HIV replication remains unknown; however, it was apparently in part a cAMP-mediated event. Using total body irradiation, the ionizing radiation has been reported to increase tissue levels of cAMP in tissue biopsies from different organs (12, 13) and in primary human fibroblasts in cell culture (14). That cAMP mediated the enhancement of HIV replication by x-irradiation is not inconceivable, since HIV infection of CD4 positive cells has been associated with sustained elevation of intracellular levels of cAMP (10), and agents that increase intracellular levels of cAMP tend to enhance HIV replication (16; M. Nokta and R. Pollard, manuscript submitted for publication). In the latter reports, forskolin, a direct activator of adenylate cyclase, and the use of the phosphodiesterase inhibitor isobutylmethylxanthine enhanced HIV replication as determined by RT activity. Moreover, the protein kinase A inhibitor, H-8, partially inhibited the x-ray-induced enhancement of HIV replication, as shown in Table IV.

Part of the replication cycle of HIV involves a double-stranded proviral DNA intermediate integrated into the cellular DNA. Thus, viral transcription and release is dependent upon cellular activation and proliferation. Cellular division has been shown to be regulated by a cAMP-dependent protein kinase A (18). Therefore, x-irradiation probably increases intracellular levels of cAMP as part of a radioprotective mechanism that involves the induction of repair enzymes. It is known that the radioprotective agent cysteamine (19) and phosphodiesterase inhibitors enhance cellular levels of cAMP and that the latter enhance HIV replication (16; M. Nokta and R. Pollard, manuscript submitted for publication). Thus, the induction of intracellular levels of cAMP and cellular repair enzymes that are involved in cellular DNA synthesis, to repair the x-ray-induced DNA breaks, would possibly facilitate HIV replication. Alternatively, DNA damage by x-irradiation may have stimulated stress responses in the cells that led to the activation of HIV. HIV gene expression was reported to be activated by ultraviolet light and by the radiomimetic compound mitomycin C; both induce DNA damage in human cells (20, 21). In addition, x-irradiation may have induced heat shock proteins (as a stress response) that in turn activated HIV. Heat shock proteins have been implicated in the induction of HIV long terminal repeat (22). A substantial sequence similarity to the heat shock response consensus sequence is found in the NF-KB binding sites in the HIV long terminal repeat (22).

The fact that protein kinase C is involved in T cell activation and proliferation, and that the latter is required for HIV replication, might suggest the potential involvement of a protein kinase C pathway in the radiation-induced HIV replication. Inhibitors of protein kinase C have been shown to inhibit the replication of HIV induced by certain stimuli, such as phorbol esters (23).

Another possible pathway in which x-irradiation

may affect cell activation and HIV replication could be through the induction of DNA hypomethylation. Recently, it was reported that γ-radiation induced the hypomethylation of DNA, which resulted in the differentiation of a neuroblastoma cell line (24). This radiation-induced hypomethylation was a consequence of decreased intranuclear levels of DNA methyltransferases. The expression of several cellular genes is reported to be regulated by the level of their methylation; these genes include adenine phosphoribesyltransferase (25), hypoxanthine phosphoribosyltransferase, silent integrated retroviral genomes (26), and thymidine kinase genes (27, 28).

Some have suggested the use of low dose total body irradiation for the treatment of HIV infection; however, the sensitivity of HIV-infected cells to x-irradiation did not differ significantly (data not shown) from uninfected cells. Also, it is noteworthy to mention that the virus released from x-irradiated cells was as infectious as that released from unirradiated cells. These results do not necessarily refute the possibility of the use of TBI in the treatment of HIV-infected patients, since higher lethal doses still may be useful if followed by bone marrow transplantation. This approach has been studied by others (29). However, it was reported that HIV was not inactivated when irradiated with very high doses (2×10^5 cGy) *in vitro* and had no effect on virion infectivity (30).

Though the data might raise the concern that patients with HIV infection may be at risk of exacerbating a latent infection (after exposure to roentgen rays during diagnostic imaging, the doses of radiation utilized in these procedures are found below those reported herein. However, zidovudine completely inhibited HIV replication in cultures that were previously irradiated (data not shown), which indicates that the released virus was sensitive to zidovudine.

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