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Productive human immunodeficiency virus infection levels correlate with AIDS-related manifestations in the patient

(radiation-resistant human immunodeficiency virus expression/AIDS pathogenesis/monitoring of antiviral therapy)

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ABSTRACT **Mononuclear cells were obtained from 71 human immunodeficiency virus type 1 (HIV-1) seropositive subjects presenting at first visit either as asymptomatic or with minor symptoms and with CD4 lymphocytes >550 per mm³ (group A, 35 patients) or as patients with AIDS, AIDS-related illnesses, or CD4 lymphocytes <400 per mm³ (group B, 36 patients). After 1–5 years of follow-up, 13 patients of group A had essentially retained their initial status (asymptomatics); the 22 others had suffered clinical or immunological deterioration (progressors). Frozen cells were thawed and submitted to lethal γ-irradiation *in vitro* (4500 rads; 1 rad = 0.01 Gy) before they were cultured with normal phytohemagglutinin-stimulated lymphocytes to determine radiation-resistant HIV expression *ex vivo* (R-HEV). HIV antigenemia correlated with R-HEV values in 142 samples ($r = 0.92$, $P < 0.001$) but was a less sensitive predictor of disease than R-HEV. R-HEV was detected in all specimens from patients with major AIDS-related illnesses or HIV-associated CD4 lymphopenia. In 77% of the progressors from group A, R-HEV detection preceded the onset of AIDS-associated disease or CD4 lymphopenia by 1 year (average). Conversely, R-HEV was low or was not detected in 36 sequential specimens from the 13 patients who remained asymptomatic over the following 2–5 years. Thus, persistently low HIV expression *in vivo* predicted a nondiseased state, whereas higher HIV expression levels seemed necessary for disease to occur. These data indicate that R-HEV is related to productive HIV infection *in vivo*, the latter acting as a determinant of AIDS-related illnesses. In view of this, measurement of HIV expression levels in the patient should be useful in antiviral efficacy trials.**

Increases in serum human immunodeficiency virus (HIV) antigen prevalence (1–8) and in retrovirus isolation rates from plasma (9–15) or from mitogen-stimulated mononuclear cells (MNCs) in coculture systems (16–21) all indirectly indicate higher HIV replication rates *in vivo* in symptomatic versus asymptomatic HIV-1-infected individuals. However, previously it had not been possible to routinely assess the frequency of HIV-infected cells actually expressing virus in the infected person (reviewed in ref. 22). Here we show that lethal γ-irradiation of a patient's MNCs *ex vivo*, prior to coculturing with normal phytohemagglutinin (PHA)-stimulated lymphocytes, yields HIV in quantities that correlate with levels of HIV expression in the patient and that these levels also correlate with AIDS-related disease in the infected individual.

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PATIENTS

The present study was undertaken as a retrospective analysis of medical records and specimens from 71 HIV-1 antibody carriers (4 females, 67 males; mean age = 36 ± 8.8 years, range 9–58) who had attended one of two out-patient clinics for possible HIV-related illness between 1984 and 1989. The patients represent a nonrandomized selection based on knowledge of clinical/immunological history.

All subjects repeatedly tested positive for HIV-1 antibodies in a recombinant HIV-1 enzyme-linked immunoassay (Abbott, HIV-1 EIA); all positives were confirmed by immunofluorescence staining (23) of human T-lymphotropic virus (HTLV)-IIIB infected, methanol-fixed H9 cells (gift from R. Gallo, National Institutes of Health) and/or in a recombinant-based competitive confirmation EIA (Abbott) and/or by standard Western blots (courtesy of C. Desgranges, Lyon, France). None received any form of antiviral therapy during studies of HIV expression levels.

Thirty-six individuals presented, with a history, past or present, of major AIDS-related disease [Centers for Disease Control (CDC) class IV, $n = 26$] or with $CD4^+$ lymphopenia (<500 per mm³, $n = 10$) (patient group B). The remaining 35 individuals (group A) initially had no major AIDS-related illnesses (CDC II/III) and had blood CD4 lymphocyte counts within normal limits (570–1575 per μl; mean = 935 ± 308). Of these 35, 22 showed disease progression, manifested by a decline in CD4 lymphocytes to <400 per μl (from 786 ± 182 to 108 ± 109), after a mean follow-up of 39.6 ± 11.7 months, concurrent with major clinical disease (progressor subgroup) ($n = 14$) or absence of disease ($n = 8$). In the 13 other subjects, progression was limited to a moderate decline in CD4 lymphocytes (from 1162 ± 337 to 804 ± 203), remaining >500 per μl as of the last visit over a mean follow-up of 33 ± 13.6 months (asymptomatic subgroup).

METHODS

MNC Separation and Characterization. Forty to 50 ml of blood was aspirated into sterile glass tubes containing 2500 international units of lithium heparinate (Vacutainer, Becton Dickinson) for the purpose of eventual virological studies after informed consent was obtained from each subject. Each individual gave blood one to five times at 8- to 13-month intervals. Serum or plasma was tested for HIV antigen using a commercially available EIA (Abbott). MNCs from Ficoll/Hypaque gradients were collected in polystyrene tubes,

Abbreviations: MNC, mononuclear cell; HIV, human immunodeficiency virus; R-HEV, radiation-resistant HIV expression *ex vivo*; PHA, phytohemagglutinin; DRB, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole; EIA, enzyme-linked immunoassay.

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frozen in 10% dimethyl sulfoxide/20% fetal bovine serum/RPMI medium, and stored in liquid nitrogen (up to 60 months) using conventional procedures. In one patient (subject 18), broncho-alveolar cells were obtained through a diagnostic lavage procedure. MNCs were washed three times in RPMI medium supplemented with antibiotics and frozen as described above.

After thawing, samples from washed MNCs (1×10^6 per ml) were incubated with fluoresceinated mouse monoclonal antibodies to CD4 or CD8 and with propidium iodide. CD4 and CD8 lymphocyte counts and the phenotypes of MNCs from Ficoll separations were obtained with automated systems (Ortho Instruments; Facscan, Becton Dickinson).

Radiation-Resistant HIV Expression *ex Vivo* (R-HEV) Assay. Freshly thawed cell suspensions were exposed to three convergent fixed sources of γ-rays (^{192}Cs) within a biological laboratory irradiator (ORIS, Gif-sur-Yvette, France, courtesy of de Schmilblitz) for a total of 4500 rads (1 rad = 0.01 Gy). Irradiated cells were then distributed to culture wells.

For each irradiated cell specimen, four replicates each of 5×10^5 and 1×10^5 cells were seeded in 2.2-ml wells (24-well plates, Nunc). PHA-stimulated lymphocytes (5×10^5) from either of two donors were added within 1 hr of irradiation to each replicate in 2 ml of fresh culture medium consisting of RPMI 1640 medium supplemented with 1% glutamine, 1% pyruvate, 70 international units of human recombinant interleukin 2 (Roussel-Uclaf, Paris, courtesy of Deraedt), and 10% (vol/vol) fetal bovine serum (Flow Laboratories). Cell mixtures were then cultivated for 10–11 days in a humidified 5% CO_2/95% air atmosphere. Cell-free supernatants and cell samples were then taken from each replicate for testing.

Culture supernatants were treated with Triton X-100 and were tested under code for HIV-1 antigen (Abbott). Supernates with signal/cutoff ratios >3 were considered virus positive. The R-HEV score for a given cell suspension was calculated as the percentage of virus-positive culture wells out of the combined two sets of four replicates (Fig. 1). As a control, intracellular p24-specific immunofluorescence on methanol-fixed cells was detected with mouse monoclonal antibodies to HIV-1 p24 (gift from Jeffrey Hunt, Abbott) and fluoresceinated goat anti-mouse IgG (Atlantic Antibodies, Scarborough, ME). When positive, this assay was always in agreement with the coded cell-free immunocapture assay.

Treatment of Irradiated Patients' Cells with Metabolic Inhibitors. MNCs from patients and fresh MNCs from one HIV-seronegative donor were irradiated (45 Gy) and incubated in supernatants from 3-day PHA-stimulated lymphocytes and fresh culture medium (vol/vol) with or without the inhibitor for 3 hr (mitomycin C, 10 μg/ml; Sigma) or 24 hr [5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB), 10 μg/ml; generous gift from U. Chen]. Cells were then washed three times, left to stand in fresh culture medium for 3 hr (for DRB experiments only), and washed once again. Eight replicates of 1.5×10^5 inhibitor-treated or untreated patient cells were added to an equal number of reciprocally untreated or treated normal irradiated cells as a control for a carry-over of the inhibitor into the indicator PHA-stimulated lymphocytes that were added to each well. HIV expression was assayed by HIV antigen detection in culture supernatants at day 11.

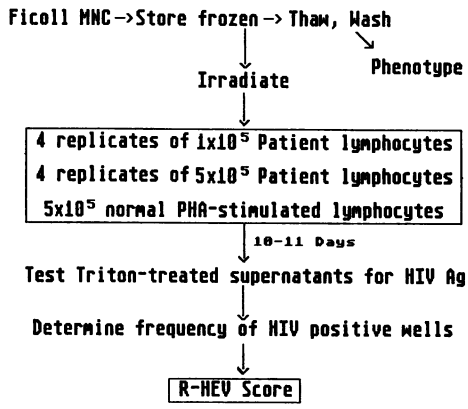

FIG. 1. R-HEV assay.

Statistical Analysis. All statistical tests performed in the study were contingency tests. χ^2 tests were used in all cases.

RESULTS

MNC Characterization. In the first available cell specimen from 10 asymptomatics, lymphocytes and monocytes represented 30% ± 10% and 9.5% ± 4%, respectively; in 11 progressors, the counts were 22% ± 6% (CD4) and 8% ± 3% (monocytes). In 22 patients from group B, these proportions were 14% ± 9% and 13% ± 7%, respectively. Viability of freshly thawed cells was in excess of 95% for all mononucleated cell types prior to irradiation.

Quantitation of Infection and Reproducibility of the R-HEV Assay. R-HEV scores for a given specimen were derived from the percentages of HIV-positive cultures from four replicates each of two cell concentrations 5-fold apart (10^5 and 5×10^5). This was chosen in preference to more conventional titration systems as a compromise between limited patient cell availability and the sensitivity of virus detection rates and quantitation. Our system recognized 5-fold differences in the frequencies of HIV-infected cells within a two logarithm scale.

To correlate R-HEV scores with the number of infected cells placed into culture, 25 irradiated cell specimens from patients were serially diluted from 5×10^5 to 4×10^3 MNCs, and 4–14 replicates of each cell dilution were cultured with PHA-stimulated lymphocytes as above. By analysis of the fraction of HIV-negative cultures in each dilution and the use of the Poisson formula as performed for the determination of frequency of rare events that conform to single-hit kinetics (24), the frequency of radiation-resistant HIV-infected cells was calculated. A linear relationship was observed (data not presented) showing that R-HEV scores of 75–100% (six to eight wells positive for HIV after irradiation) corresponded to one HIV-infected cell in $6 \pm 3 \times 10^4$ MNCs, whereas R-HEV scores of 12–25% (one or two in eight wells positive for HIV after irradiation) corresponded to one HIV-infected cell in 10^6 MNCs. Thus, negative R-HEV scores would represent one or less HIV-infected cell in 3×10^6 MNCs, the lower limit of sensitivity of this procedure.

Reproducibility of the R-HEV assay was evaluated by testing 31 patient specimens twice, with the repeat test done 1–10 months later. The mean coefficient of variation for R-HEV scores was 10%.

Effect of Metabolic Inhibitors on R-HEV. R-HEV was totally abolished when initiation of RNA synthesis was impaired by treatment of patients' cells with DRB during the 24 hr before amplifier lymphoblasts were added (Table 1). HIV expression from irradiated cells treated with the DNA synthesis inhibitor mitomycin C was not significantly different from that of irradiated patients' cells not treated with the inhibitor (Table 1).

R-HEV Scores, Serum HIV Antigen, and Clinical/Immunological Status. One hundred forty-two cell specimens were stratified according to the frequencies of virus-positive replicates following irradiation and cocultivation, from 0 positive out of eight replicates (R-HEV scores = 0), to eight in eight positive (R-HEV scores = 100). This was then correlated with the prevalence of HIV antigen detection in the sets of the corresponding paired sera (Fig. 2). The frequency at which HIV antigen was detected in serum was

Table 1. Effect of inhibition of RNA synthesis initiation or of DNA synthesis on HIV expression by irradiated patients' cells

Mixture of irradiated cells	DRB	% HIV positive A	% HIV positive B	% HIV inhibition	Mitomycin C	% HIV positive C	% HIV positive D	% HIV positive E	% HIV inhibition
Patient/normal	−/−	100	100	0	−/−	88	100	100	0
Patient/normal	+/−	0	0	100	+/−	75	100	75	19
Patient/normal	−/+	100	100	0	−/+	50	75	75	38

After γ-irradiation, MNCs were incubated with (+) or without (−) the inhibitor (3 hr with mitomycin C; 24 hr with DRB) and washed; eight replicates of 1.5×10^5 inhibitor-treated cells were added to an equal number of untreated cells in reciprocal mixtures to which 5×10^5 amplificator cells were added. Detection of p24 antigen (signal/cutoff × 3) at day 10 determined HIV-positive cultures. Results are expressed as mean percentages of HIV expression inhibition in reference to inhibitor-untreated patient/normal cell mixtures. Patients are coded A–E.

found to increase significantly in proportion to the frequency of radiation-resistant HIV-positive cultures ($r = 0.92$, $P < 0.001$).

HIV antigen, when detectable in serum ($n = 63$), always corresponded to cells with a positive R-HEV score except for one instance in which the R-HEV-positive sample was from alveolar MNCs rather than from the concurrent blood MNCs (patient 18). HIV antigen-negative sera (85 specimens) corresponded to 39 R-HEV-negative specimens and 46 R-HEV-positive cell specimens. The latter were from 3 asymptomatics (5 specimens), 14 progressors (24 specimens), and 13 subjects from patient group B (17 specimens). In total, serum HIV antigen was *not* detected in 27 of 58 subjects at a time when they presented with major clinical disease or/and lymphopenia, whereas all were R-HEV positive. HIV antigen detection in individuals with minor symptoms and normal CD4 lymphocyte counts at first visit predicted progression in seven of seven progressors with this marker. The absence of HIV antigen in serum predicted a nonprogressive course in only 48% of 27 patients from group A.

All 36 subjects belonging to patient group B tested positive for R-HEV as did 21 in 21 progressors tested at a time when they had developed CD4 lymphopenia or disease. In the latter subgroup, R-HEV scores increased with progression to disease in sequential specimens (Table 2). Ten of 13 asymptomatics remained negative for R-HEV with two to four samples tested per individual over 27.2 ± 9.4 months. Two asymptomatics had low positive R-HEV scores (15% ± 6%) in two specimens each, and a third had three R-HEV negative specimens over 16 months followed by one low-level positive at 24 months. In group A, of the 19 individuals who initially tested positive for R-HEV, 2 remained asymptomatic and 17 progressed. The predictive value sensitivity for progressive disease of a R-HEV-positive specimen at first visit was 77%, with a specificity of 85%. Of the 16 individuals with negative R-HEV specimens at first visit, 5 eventually progressed to disease, in parallel with conversion to positive R-HEV scores.

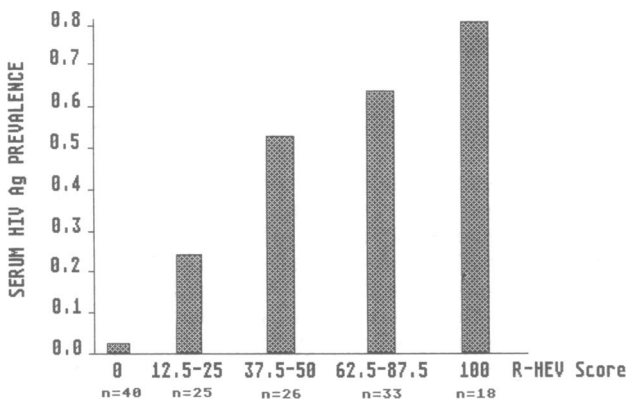

FIG. 2. Correlation between cell-associated R-HEV scores and serum HIV antigen detection rates. One hundred forty-two cell specimens tested for radiation-resistant HIV expression are stratified in five sets according to R-HEV score ranges and plotted against the prevalence of HIV antigen detection in the corresponding serum specimen. The frequency at which HIV antigen was detected in serum increased in proportion to the frequency of positive cell replicates ($P < 0.001$).

CD4 lymphocytes were measured in 142 samples and then compared with the corresponding R-HEV score. Decrease in CD4 counts were significantly associated ($P < 0.001$) with R-HEV scores ≥50% (Fig. 3 *Upper*). Conversely, increasing CD4 counts correlated with increasing prevalence of negative R-HEV scores (Fig. 3 *Lower*, $P < 0.001$). R-HEV scores were significantly higher ($P < 0.01$) in 12 patients with AIDS opportunistic infections (mean R-HEV = 82% ± 14%) and low CD4 lymphocyte counts (67 ± 44 per mm^3) than in 28 asymptomatics (mean R-HEV = 14% ± 18%) with CD4 counts above 650 per μl (1018 ± 283 per μl). Twelve patients with Kaposi sarcoma and no major immunodeficiency (CD4 = 654 ± 253 per μl) had R-HEV scores ≥50% significantly more often ($P < 0.02$) than 34 CD4-matched (683 ± 188 per μl) HIV carriers with no or minor symptoms.

In one patient (subject 18), radiation-resistant HIV-infected cells in blood were <1 in 10^6 MNCs (R-HEV score = 0), whereas in concurrent broncho-alveolar cells, the frequency of infection was 1 in 3×10^4 irradiated MNCs. Patient 18 was presenting with a clinically severe interstitial lymphocytic pneumonia at the time.

DISCUSSION

Justification for γ-Irradiation. *In vitro* mitogenic stimulation in lymphocyte cocultures used to assess HIV load up-regulates HIV expression (25–28) and may lead to overestimates of actual HIV expression levels. Because γ-irradiation arrests cell division and leads to cell death (here, in 1–3 days), it should be able to interfere with HIV expression in latently infected cells. γ-Irradiation at 4500 rads diminished HIV expression from patients' cells by 5- to 500-fold relative to that of the same unirradiated specimens ($n = 140$ specimens, data not presented), in contrast to ultraviolet irradiation, which enhance HIV expression through HIV long terminal repeat-directed RNA transcription (29, 30). The fraction of HIV-infected cells that expresses virus *ex vivo* only if mitogenically triggered (the radiation-sensitive set) might not be as relevant to pathogenicity as those cells actively expressing HIV (31), as suggested here by patient 18: radiation-sensitive HIV-infected cells were 100 times more frequent in blood than in pathological lungs at a time of a major HIV-related pulmonary disease (lymphocytic pneumonia) and no AIDS-related systemic symptoms.

γ-Irradiation did not block retrovirus propagation from cells already producing virus. In our hands, *one* lethally irradiated HIV-1-infected H9 cell, a malignant T cell, propagated virus for at least 24 hr, as did similarly treated HIV-infected, PHA-driven normal lymphocytes (unpublished observations); production of HIV by irradiated cells

Table 2. Virological and immunological markers in HIV-infected patients

Patient group	First specimen			Subsequent specimen		
	A (Asymp)	A (Prog)	B	A (Asymp)	A (Prog)	B
Specimens/patients	13/13	22/22	37/37	26/13	36/21	16/12
Mean R-HEV*	2 ± 6	24 ± 2	62 ± 24	2 ± 6	60 ± 32	64 ± 27
R-HEV, ≥50% prevalence	0	0.18	0.78	0	0.69	0.75
R-HEV, null prevalence	0.85	0.11	0	0.85	0.05	0
HIV antigenemia, null prevalence	1.00	0.67	0.35	0.96	0.42	0.25
Blood CD4*	1162 ± 256	801 ± 176	305 ± 300	980 ± 365	356 ± 209	407 ± 271
Blood CD4/CD8 ratio*	1.11 ± 0.54	0.61 ± 0.36	0.32 ± 0.19	0.96 ± 0.28	0.47 ± 0.28	0.38 ± 0.18
Months at follow-up* (range)				25.6 ± 10.2 (10–46)	24 ± 7.9 (12–38)	9.8 ± 7.2 (3–20)

For patient description, see *Patients*. Asymp, asymptomatics; Prog, progressors.
*Values are presented as mean ± SD.

depended on newly transcribed HIV RNA *ex vivo* (DRB experiments, Table 1): DRB interferes with the formation of the RNA initiation complex but does not affect on-going RNA elongation or termination processes (32). Thus, patients' cells propagating HIV after irradiation would be engaged into active HIV expression *in vivo*, maintaining this activity for 1–3 days *ex vivo*.

The major R-HEV-contributing cells should be found among CD4 lymphocytes and/or monocytes in the process of expressing HIV *in vivo*. If only ascribed to patients' CD4 lymphocytes, the frequency of cells expressing HIV in our system would match those reported by others in advanced disease with CD4-sorted, HIV RNA-expressing lymphocytes

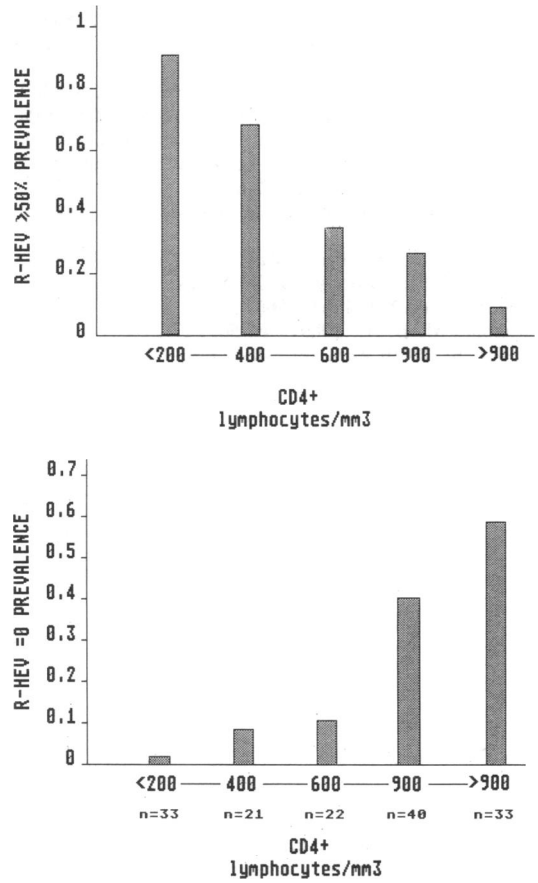

FIG. 3. Correlation between blood $CD4^+$ counts and R-HEV scores. The prevalence of R-HEV scores ≥50% (*A*) or of R-HEV scores = 0 (*B*) is plotted against blood $CD4^+$ lymphocytes stratified in five sets (see abscissa). $CD4^+$ cell depletion correlates with HIV expression levels in the R-HEV test.

(33): 1 in 690 ± 190, in limiting dilution analysis of MNCs from three patients with AIDS-opportunistic infections and blood CD4 counts below 50 per μl.

Relevance of R-HEV to *in Vivo* HIV Expression Levels and Patients' Status. HIV production by MNCs, following γ-irradiation and amplification with normal PHA-stimulated lymphocytes, correlated with the detection of HIV antigen in serum (Fig. 2, $P < 0.001$). Such a level of correlation was not found when unirradiated cells were used (data not presented). HIV isolation and quantitation with our system were thus relevant to actual *in vivo* HIV replication. However, R-HEV showed higher sensitivity than serum HIV antigen: the latter was not detected in one-third of individuals presenting with major disease and/or CD4 lymphopenia, as reported (34–37), whereas all had a R-HEV-positive cell specimen.

HIV expression levels measured by R-HEV appeared to determine whether disease or nondisease was or would be present: low or negative R-HEV scores, corresponding to low frequencies of replication *in vivo*, were associated with no disease or minor clinical or immunological disease for as long as HIV expression remained at such levels (asymptomatics), whereas higher HIV replication rates preceded or coincided with major disease or CD4 lymphopenia (progressors and group B patients). That HIV expression may precede morbidity (including CD4 cell depletion) speaks for active HIV infection to be cause of disease, not an opportunistic consequence of an otherwise unexplained immune deficiency (38).

Productive infection seemed permanent rather than intermittent in sequential specimens from progressors and from patients with disease (Table 2). Of note, persistent HIV replication occurred in the presence of relatively high numbers of $CD4^+$ and $CD8^+$ cells *in vivo* and *in vitro* (patients with Kaposi, progressors, patient 18), suggesting that cells expressing HIV manage in some way to circumvent the T-lymphocyte surveillance system (39, 40).

In conclusion, our assay system provided reproducible estimates on HIV replication rates actually occurring in HIV-1-infected subjects, the levels of which were linked to AIDS-related disease. Sequential estimates of radiation-resistant HIV infectious cells produced *ex vivo* by γ-irradiated MNCs should help determine the efficacy of various regimens and combinations of antivirals (41), possibly as of their phase I studies. In preliminary studies, R-HEV scores were significantly transiently altered in patients receiving treatment with 3'-azido-3'-deoxythymidine (unpublished data).

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