# Factors Underlying Spontaneous Inactivation and Susceptibility to Neutralization of Human Immunodeficiency Virus

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Received March 5, 1992; accepted May 21, 1992

To determine the factors governing inactivation and neutralization, physical, chemical, and biological assays were performed on a molecular clone of human immunodeficiency type 1 (HIV-1HXB3). This included quantitative electron microscopy, gp120 and p24 enzyme-linked immunosorbent assays, reverse transcriptase assays, and quantitative infectivity assays. For freshly harvested stocks, the ratio of infectious to noninfectious viral particles ranged from 10<sup>-4</sup> to 10<sup>-7</sup> in viral stocks containing 10<sup>9</sup> to 10<sup>10</sup> physical particles per milliliter. There were relatively few gp120 knobs per HIV particle, mean  $\approx$  10 when averaged over the total particle count. Each HIV particle contained a mean  $\approx$  5  $\times$  10<sup>-17</sup> g of p24 and  $\approx 2 \times 10^{-16}$  g of RNA polymerase, corresponding to about 1200 and 80 molecules, respectively. The spontaneous shedding of gp120 envelope proteins from virions was exponential, with a half-life  $\approx$  30 hr. The loss of RNA polymerase activity in virions was also exponential, with a half-life  $\approx$  40 hr. The physical breakup of virions and the dissolution of p24 core proteins were slow (half-life > 100 hr) compared to the gp120 shedding and polymerase loss rates. The decay of HIV-1 infectivity was found to obey superimposed single- and multihit kinetics. At short preincubation times, the loss of infectivity correlated with spontaneous shedding of gp120 from virions. At longer times, an accelerating decay rate indicated that HIV requires a minimal number of gp120 molecules for efficient infection of CD4+ cells. The blocking activity of recombinant soluble CD4 (sCD4) and phosphonoformate (foscarnet) varied with the number of gp120 molecules and number of active RNA polymerase molecules per virion, respectively. These results demonstrate that the physical state of virions greatly influences infectivity and neutralization. The knowledge gained from these findings will improve the reliability of in vitro assays, enhance the study of wild-type strains, and facilitate the evaluation of potential HIV therapeutics and vaccines. © 1992 Academic Press, Inc.

#### INTRODUCTION

Human immunodeficiency viruses (HIV) are structurally and functionally labile. Four mechanisms are known to contribute to this property. These include the spontaneous dissociation of gp120 proteins from the viral envelope (Gelderblom et al., 1985; Modrow et al., 1987), the physical breakdown of the lipid membrane covering viral particle (Nara et al., 1987a), the dissolution of p24 core proteins encasing viral RNA (McDougal et al., 1985), and the spontaneous loss of RNA polymerase activity (Lori et al., 1988; Goff, 1990). At present, there is little information on the kinetics of these chemical reactions, their rate constants, and how these reactions influence the activities of antiviral agents that attack extracellular or intracellular events in the viral life cycle (Looney et al., 1990; Lavne and Dembo, 1992; Lu et al., 1992). Furthermore, there is

scant knowledge of how widely these rates vary between laboratory strains and wild-type isolates under physiologic conditions. To start elucidating such issues, we undertook an extensive study of these basic reactions with HIV-1HXB3, a molecularly cloned laboratory strain (Shaw *et al.*, 1984). HIV-1HXB3 was chosen over others because it was easy to grow and store in large volumes. Also, recombinant gp120, p24, and RNA polymerase proteins were available for the absolute calibration of chemical assays.

The strategy of this study was to perform a variety of physical, chemical, and biological assays on the same viral stock, permitting correlations between the different types of data. Physical measurements of the actual number of particles and the number of gp120 knobs per particle were carried out by electron microscopy (EM). Quantitative determinations of gp120 envelope and p24 core proteins were carried out by ELISA. Biochemical measurements of RNA polymerase activity were performed by quantitative enzymatic assays. Biological measurements of viral titer, spontaneous viral

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inactivation, and the activities of antiviral agents were performed by quantitative infectivity assays with transformed cells and primary blood mononuclear cells. To determine basic kinetic parameters, such as half-lives and reaction orders, assays were repeated on viral stocks that were preincubated for various time intervals at 37°. To investigate how the perturbation of virus-cell binding was influenced by thermal treatment, sCD4 was used as a blocking agent at concentrations not facilitating spontaneous shedding of gp120 (Moore et al., 1990; Hart et al., 1991; Layne et al., 1991; Dimitrov et al., 1992). To investigate how virion-associated reverse transcriptase activity influenced infectivity, trisodium phosphonoformate (foscarnet) was used as an inhibitor of viral RNA polymerase (Majumdar et al., 1978; Öberg, 1983).

Previous studies found that the blocking activity of sCD4 was inversely related to the density of CD4<sup>+</sup> cell in viral infectivity assays (Layne et al., 1991). For "unsaturated" assays at low target cell densities, the biological blocking activity of sCD4 corresponded to the gp120-sCD4 association constant (Kassoc) from chemical measurements. That is, the inhibition of infection was proportional to the formation of gp120-sCD4 complexes (Layne et al., 1990). For "saturated" infectivity assays at high target cell densities, on the other hand, the biological blocking activity of sCD4 fell far below the chemical  $K_{assoc}$ . This decline occurred because the CD4 receptors on cell surfaces, which mediated infection, competed successfully with the sCD4 molecules in solution for viral-associated gp120 molecules (Layne et al., 1989, 1991). To permit direct comparisons between chemical and biological measurements, the sCD4 blocking assays in this study were carried out at low target cell densities.

Also, previous studies indicated that HIV requires a minimal number of free (unblocked) gp120 molecules for efficient infection of CD4<sup>+</sup> cells (Byrn *et al.*, 1989; Layne *et al.*, 1990). When more than this minimal number were present on a virion, the rate of infection was proportional to free gp120. When less than this number were present, the rate of infection was significantly reduced. These studies did not, however, estimate the actual size of this threshold. Further goals of this study were to determine the minimal number of gp120s for HIV-1HXB3 by utilizing the relevant physical, chemical, and biological data and to see whether this number varied for primary and transformed human CD4<sup>+</sup> cells.

# MATERIALS AND METHODS

#### Viral stocks

H9 cell cultures (American Type Culture Collection, CRL 8543) were inoculated with HIV-1HXB3 as previ-

ously described (Layne *et al.*, 1991). After 2 days, infected H9 cultures (1000 ml total volume) were clarified by centrifugation ( $20^{\circ}$ , 20 min, 10,000 g) and frozen ( $-70^{\circ}$ ) in 10-ml aliquots. Also to monitor and optimize the titer of HIV-1HXB3 stocks, infected H9 cultures were incubated for periods of 4 to 6 days. At daily intervals, 5-ml aliquots of supernatant were removed, clarified by centrifugation, and frozen. Subsequently, these samples were assayed in parallel for infectious units.

### Quantitative infectivity assays

Human peripheral blood mononuclear cells (H-PBMC) were isolated by centrifugation through FicoII– Paque (Pharmacia). H-PBMC were washed twice [suspended in 50 ml of phosphate-buffered saline (PBS) and centrifuged for 10 min at 300 *g*], red cells were disrupted with 25 ml of ammonium chloride lysis buffer (Biofluids, Inc.), and H-PBMC were again washed twice. H-PBMC were cultured (37°, 5% CO<sub>2</sub>) in media [RPMI 1640 and 10% fetal bovine serum] containing 2  $\mu$ g ml<sup>-1</sup> phytohemagglutinin. After 2 days, nonadherent cells were centrifuged and placed in fresh media containing 32 units ml<sup>-1</sup> of IL-2. The next day, these stimulated H-PBMC were used as target cells.

H-PBMC target cells, and CEM-SS target cells as previously described (Nara et al., 1987b; Layne et al., 1990, 1991), were suspended in 50 nM DEAE-dextran for 30 min. After the DEAE-dextran was removed, a fixed number of target cells were added to 2-ml microcentrifuge tubes containing media with different sCD4 concentrations. To hold the inoculum-to-volume ratio constant, identical aliguots of HIV stock were added to each tube. Particular ratios (either 10 or 20%) were selected to give an m.o.i. less than 0.1, a total gp120 concentration less than 0.1  $\times$  K<sub>assoc</sub>, and a satisfactory statistical count of infectious units. Tubes were placed on a roller apparatus during the 1-hr infection period at 37°. To remove cell-free virus and sCD4, infected target cells were washed (1 min at 10,000 g, suspended in 1 ml of PBS and centrifuged) and suspended in fresh media. Indicator cell monolayers were made by adding  $3.5 \times 10^4$  CEM-SS cells to microplate wells as previously described (Layne et al., 1990, 1991). Infected cell monolayers were made by adding a small number (from 1250 to 5000) of H-PBMC or CEM-SS target cells to indicator cell monolayers. Eight replicate wells were plated per time point and sCD4 concentration. Syncytial forming units (SFU) representing the infection of individual target cells by cell free virus were counted 3 to 5 days following plating (Nara et al., 1987b; Nara and Fischinger, 1988).

#### Viral decay assays

Frozen HIV-1HXB3 stock (250 ml) was thawed, pooled, and incubated with gentle mixing at 37°. At

regular intervals (1 to 2 hr), quantitative infectivity assays were performed with H-PBMC and CEM-SS target cells ( $1.4 \times 10^4$  cells ml<sup>-1</sup>, 1.8-ml reaction volumes, and 10% inoculum-to-volume ratio) in media containing 0 and 0.5 n*M* sCD4. Also at regular intervals (4 or 12 hr), a "prespin" sample of viral stock was frozen (-70°) for subsequent assays. At the same time, 8-ml aliquots of stock were ultracentrifuged (20°, 40 min, 155,000 *g*) and the resulting "postspin supernatants" were frozen for subsequent assays. After all remaining supernatant was swabbed from the ultracentrifuge tubes, viral "pellets" were suspended in fresh media and frozen for subsequent assays.

#### gp120 ELISA

Frozen samples from viral decay assays were thawed and lysed with Nonidet P40 (NP40), final concentration 0.5%. Immulon-II microplate wells (Dynatech) were coated with D7324 capture antibody (Aalto Bioreagents), washed, and blocked as previously described (Moore and Jarrett, 1988; Moore et al., 1989). Next, 100  $\mu$ l of each sample was added to six replicate wells and incubated for 3 hr at room temperature. Unbound protein was removed by washing twice with 200  $\mu$ l of solution containing 144 mM NaCl and 25 mM Tris (pH 7.6). Bound gp120 was detected with a pool of HIV-1-positive human serum, an alkaline-phosphatase-conjugated antibody to human IgG (SeraLab), and an AMPAK ELISA (Novo Nordisk) amplification system (Moore et al., 1990). Reactions were stopped with  $50 \mu l$ of 0.5 M HCl and optical densities at 492 nm were measured. Protein concentrations were determined against recombinant HIV-1111B gp120 derived from CHO cells (Celltech). The purity (>95%) and concentration of the recombinant protein were measured by SDS electrophoresis and amino acid analysis, respectively (data not shown). The gp120 standards were included on all microplates (three replicates in serial threefold dilutions).

## p24 ELISA

Frozen samples from decay assays were thawed and lysed with Triton X-100, final concentration 0.5%. One hundred microliters of each sample was added to four replicate wells in commercial microstrips (NEK-060A, DuPont). All subsequent steps were performed in accordance with the manufacturer's instructions. The optical density (492 nm) of developed wells was determined by an automated plate reader (Vmax Kinetics, Molecular Devices). Protein concentrations were determined against recombinant HIV-1IIIB p24 supplied with the ELISA kits. p24 standards were included on all microplates (three replicates in serial twofold dilutions).

#### Quantitative RNA polymerase activity assays

Frozen samples from decay assays were thawed and lysed with Triton X-100, final concentration 0.5%. Six microliters of each sample was added to four replicate wells containing 20  $\mu$ l of 50 mM Tris (pH 7.6), 100 mM NaCl, 6 mM MgCl<sub>2</sub>, 10 mM dithiothreitol, 4  $\mu$ g ml<sup>-1</sup> oligo(dT)<sub>12-18</sub>, 40 µg ml<sup>-1</sup> poly(A), 0.12 mCi ml<sup>-1</sup> [<sup>3</sup>H]TTP, and 0.25% NP40 (Baltimore and Smoler, 1971; Goodman and Spiegelman, 1971). The microplates were then incubated for 1 hr at 37°. Next, 150 µl of stop solution containing 40 mM sodium pyrophosphate and 15 mM NaCl was added. Radioactive products were then precipitated with 20  $\mu$ l of 60% trichloroacetic acid (TCA) on ice for 30 min. The 196  $\mu$ l in each well was transferred (Micro Cell Harvester, Skatron) to  $0.45 \mu$ m filter papers with 5.0 ml of wash solution containing 6% TCA and 40 mM sodium pyrophosphate. Filter papers were dried, placed in scintillation fluid, and counted for beta activity (LKB Betaplate, Model 1205). Viral RNA polymerase activities were quantified against recombinant HIV-1111B polymerase derived from Escherichia coli (p66/p51 heterodimer, American Bio-Technologies). The purity (>95%) and concentration of the recombinant protein were measured by SDS electrophoresis and amino acid analysis, respectively (data not shown). Recombinant standards were included on all microplates (four replicates in serial twofold dilutions). For 2.0  $\times$  10<sup>-8</sup> and 1.6  $\times$  10<sup>-10</sup> g of recombinant protein, beta counts were  $4.8 \times 10^5$  and 3 imes 10<sup>3</sup>, respectively. The total counts were proportional to the protein weight and background counts were approximately  $1 \times 10^3$ .

#### RNA polymerase inhibition assays

Trisodium phosphonoformate (foscarnet) was used as a noncompetitive enzymatic inhibitor of RNA polymerase (Majumdar et al., 1978; Öberg, 1983). This antiviral agent was selected over others because the parent compound was active, readily penetrated cells, and was nontoxic at concentrations of less than 500  $\mu M$  (Helgstrand *et al.*, 1978; Sandstrom *et al.*, 1985). Prespin samples with preincubation times of 0, 8, 16, and 24 hr were thawed. Quantitative infectivity assays were carried out on these four samples with 0, 25, 50, and 100  $\mu M$  phosphonoformate in the media. To ensure that phosphonoformate was in equilibrium in these assays, it was added to the target cells 4 hr prior to adding the viral inoculum. After a 1-hr infection period, CEM-SS target cells (5  $\times$  10<sup>4</sup> ml<sup>-1</sup>) were washed, suspended in media, and added to indicator cell monolayers. For all steps, the media contained the same concentration of phosphonoformate. To evaluate the side effects of phosphonoformate on syncytia formation, three tubes without phosphonoformate in the media were inoculated with prespin samples without preincubation. Subsequently, the indicator cell monolayers contained 25, 50, and 100  $\mu$ M phosphonoformate.

# Quantitative electron microscopy

At regular intervals (4 or 12 hr) during the viral decay assays, 9.9-ml aliquots of viral stock were thoroughly mixed with 0.1 ml of polystyrene spheres (1490  $\pm$  40 Å diam from Duke Scientific or 2500 ± 130 Å diam from Polysciences), resulting in  $1 \times 10^9$  spheres ml<sup>-1</sup>. An 8-ml aliguot of this mixture was ultracentrifuged (20°, 40 min, 155,000 g), the supernatant was decanted, and the pellet was fixed with 2.5% glutaraldehyde. Next, the glutaraldehyde-fixed pellets from the decay assays (containing cosedimented beads and virus particles) were embedded in Epon either in the tips of the centrifuge tubes (in situ) or after being divided into pieces using standard procedures (Gelderblom et al., 1987). For contrast enhancement, in particular to improve the detectability of the viral glycoprotein knobs in the electron micrographs, the specimens were postfixed (60 min, 4°) with osmium tetroxide, treated (30 min, 4°) with 0.1% tannic acid (No. 1764, Mallinckrodt), and stained en bloc with 2% uranyl acetate (90 min, 4°). Sections of about 50-nm thickness were mounted on 300-mesh copper grids, poststained with lead citrate (Venable and Coggeshall, 1965), and evaluated in Zeiss electron microscopes EM902 or EM10. From pellets embedded in situ, ultrathin sections were cut at different depths of the pellet to evaluate the distribution of beads and virus particles. From the pellets divided before embedding, two to three individual pieces were similarly evaluated. From one particular depth of the in situ or one piece of the divided pellets, 10 to 15 randomly selected regions were photographed at  $12,000 \times$  and  $30,000 \times$  magnification, strictly avoiding the overlap of consecutive sections. For each micrograph, the total numbers of virus particles, polystyrene spheres, and gp120 knobs per virus particle were counted. To avoid bias, the pellets were identified by a code, which was broken only after the evaluation of micrographs. For subsequent statistical analyses of the data, only micrographs with more than five spheres and five virus particles were used.

#### Statistical analysis of data

"Normalized SFU" was defined as the mean number of SFU without sCD4 in the media divided by the mean

number of SFU with sCD4 in the media (Layne et al., 1989). To facilitate the analysis of sCD4 blocking, the slope of a normalized SFU versus sCD4 concentration plot, [(normalized SFU) - 1]  $\div$  (sCD4 concentration), was defined as the "biological blocking activity" of sCD4 (Layne et al., 1991). Biological blocking activities have units of inverse molar  $(M^{-1})$ , which permits comparison to gp120-sCD4 association constants derived from chemical measurements (Layne et al., 1990). Biological blocking activities were calculated by minimizing  $f(a, b) = \sum \{ [y_i - (ax_i + b)] \div \sigma_i \}^2$ , which gave a weighted least-squares fit to the data. y, was the mean value of 1/SFU at the *i*th sCD4 concentration,  $\sigma_i$  was the standard deviation of 1/SFU, and x<sub>i</sub> was the sCD4 concentration at the *i*th data point. For normalized SFU plots, the weighted least-squares fit gave biological blocking activities (slopes) of  $a \div b$ . Half-lives and doubling times were calculated by minimizing f(a, b). In this case,  $y_i$  was the mean value of  $\log_{10}(SFU)$  at the *i*th preincubation time,  $\sigma_i$  was the standard deviation of  $\log_{10}(SFU)$ , and x, was the preincubation time at the *i*th data point. For log-linear plots, the weighted leastsquares fit gave a half-life (or doubling time) and intercept of a and b, respectively. Unweighted leastsquares fits were obtained by setting  $\sigma_i = 1$ . The 95 and 68% confidence limits were calculated by a standard bootstrap algorithm (Efron and Tibshirani, 1991).

#### RESULTS

#### Replication characteristics of HIV-1HXB3

Figure 1 shows the exponential replication of infectious HIV-1HXB3 from two separate H9 cultures. The cultures were prepared with the same continuous H9 cell line, inoculated with the same frozen viral stock and m.o.i., and rapidly harvested by the same method (Layne et al., 1991). For the first 24 hr, the first culture (□) produced new virus at a rapid exponential rate (doubling time  $\approx$  2 hr). From 24 to 72 hr, however, the replication rate of this culture decreased and after 72 hr, the viral titer declined. For the first 96 hr, the second culture (O) produced new virus at a somewhat slower exponential rate (doubling time  $\approx$  7 hr). For this culture, samples were not taken for growth times longer than 96 hr. Thus, the inevitable effects of target cell cytolysis and depletion on viral expression were not observed. These two sets of data were selected for presentation because they bracketed typical doubling times and durations of exponential growth. Since H9 cultures always produced HIV-1HXB3 exponentially for the first 48 hr in these studies (data not shown), all viral stocks used in this study were harvested after 48 hr of viral replication.



Fig. 1. Exponential growth of infectious HIV-1HXB3 in two separate H9 cultures ( $\Box$  and O). At 24-hr intervals, aliquots of supernatant were removed, clarified by low-speed centrifugation, and frozen. Subsequently, the frozen samples were thawed and assayed for SFU. Each time point is an average of eight replicate microtiter wells. Error bars signify  $\pm 1$  SD. Doubling times are unweighted least-squares fits to the data.

### Effects of ultracentrifugation on viral infectivity

To determine the effects of ultracentrifugation on HIV-1HXB3 infectivity, a viral stock was preincubated at 37°. An 8-ml aliquot of stock was ultracentrifuged (20°, 40 min, 155,000 *g*), and the resulting postspin supernatant and pellet were assayed for SFU, along with a prespin sample of viral stock. Table 1 shows the results of this procedure at three preincubation times. The complete recovery of SFU in the viral pellets was independent of preincubation time. In addition, at all preincubation times, no SFU were detected in postspin supernatants. This demonstrated that manipulations such ultracentrifugation and suspension did not per-

turb HIV-1HXB3 infectivity and that sedimentation of HIV-1HXB3 was independent of viral aging.

#### Quantifying virion density by electron microscopy

The absolute number of HIV particles was determined by adding a known concentration of latex spheres to viral stocks and then ultracentrifuging the mixture. Since spheres and virions had similar sedimentation coefficients,  $\sim 1000$  S (Sharp and Beard, 1950; Anderson, 1968; Salmeen et al., 1976), they migrated to the bottom of the centrifuge tube in equal proportion. With thin-section electron microscopy (Fig. 2a), the ratios of spheres to virions in the pellets were evaluated. Table 2 summarizes the results of this procedure for an HIV-1HXB3 stock used throughout this study. At all four preincubation times, the average particle density of  $3.7 \times 10^{10}$  ml<sup>-1</sup> was constant to within experimental uncertainty. Three other HIV-1HXB3 stocks grown similarly had particle densities ranging from  $1 \times 10^9$  to  $5 \times 10^9$  ml<sup>-1</sup> (summarized in Table 4). For these additional stocks, the densities remained constant for preincubation times ranging from 24 to 60 hr (data not shown). These results demonstrated that the lipid membrane covering viral particles and cores were stable (at 37°) compared to other decay processes.

### Quantifying gp120 knob loss by electron microscopy

The number of gp120 knobs on cell-free virions was evaluated by thin-section electron microscopy (Fig. 2b). Individual virions were examined on EM negatives and the total number of visible knobs was recorded. A total of 20 to 30 negatives were evaluated at each preincubation time, resulting in the examination of 100 to 500 virions per time point. Figure 3 shows the results of this procedure for one EM evaluation that was particularly successful. The straight line fit to the data (solid line) had a correlation coefficient of 0.99, half-life

EFFECTS OF ULTRACENTRIFUGATION ON INFECTIVITY AS A VIRAL STOCK AGES						
Prespin SFU (mean ± SD)	Supernatant SFU (mean ± SD)	Pellet SFU (mean $\pm$ SD)	Pellet ÷ prespin ratio (mean ± SD)			
459 ± 17	0	$468 \pm 45$	$1.02 \pm 0.14$			
279 ± 13	0	234 ± 19	$0.84 \pm 0.11$			
$83 \pm 6$	0	78 ± 7	$0.94 \pm 0.15$			
	EFFECTS OF ULTRA Prespin SFU (mean $\pm$ SD) 459 $\pm$ 17 279 $\pm$ 13 83 $\pm$ 6	EFFECTS OF ULTRACENTRIFUGATION ON INFECTIVITY ASPrespin SFU (mean $\pm$ SD)Supernatant SFU (mean $\pm$ SD)459 $\pm$ 170279 $\pm$ 13083 $\pm$ 60	EFFECTS OF ULTRACENTRIFUGATION ON INFECTIVITY AS A VIRAL STOCK AGESPrespin SFU (mean $\pm$ SD)Supernatant SFU (mean $\pm$ SD)Pellet SFU (mean $\pm$ SD)459 $\pm$ 170468 $\pm$ 45279 $\pm$ 130234 $\pm$ 1983 $\pm$ 6078 $\pm$ 7			

TABLE 1

*Note.* CEM-SS target cell density was  $1.6 \times 10^5$  ml<sup>-1</sup>. SFU were averaged from eight microtiter wells. After the preincubation period, the processing times (from starting ultracentrifugations to inoculating infectivity assays) ranged from 105 to 110 min. At 9 h of preincubation, the spontaneous decay of viral infectivity was quite rapid (for example, see Fig. 7A). Thus, small differences in processing times can lead to large differences in prespin and pellet SFU.



Fig. 2. Electron micrographs of ultrathin sections of cosedimented HIV and latex spheres. (a) Micrograph showing a typical distribution of HIV and latex spheres (arrows), along with membranous and macromolecular debris. Calculating the viral particle to sphere ratio from multiple micrographs gave the absolute viral particle concentration (Table 2). Magnification was 40,000× and bar equaled 1 µm. (B) At higher magnification (86,000×), some HIV particles clearly showed envelope knobs (arrows). Evaluating the number of knobs with preincubation time permitted measurement of the gp120 shedding rate. The bar equals 100 nm.

of 57 hr, and 95% confidence limits of 38 to 144 hr (dotted lines). Counting the loss of viral-associated knobs was not precisely equivalent to measuring the chemical loss of viral-associated gp120 (data shown

#### TABLE 2

QUANTIFYING PHYSICAL PARTICLE CONCENTRATIONS BY ELECTRON MICROSCOPY

Preincubation time (hr)	Number of EM negatives evaluated	Particle to sphere ratio (mean $\pm$ SD)	Particle density (ml <sup></sup> ) (mean ± SD)
0 4 12 36	17 4 4 13	$29 \pm 10$ $42 \pm 14$ $42 \pm 5.3$ $42 \pm 5.3$ $37 \pm 16$	$\begin{array}{c} (2.9 \pm 1.0) \times 10^{10} \\ (4.2 \pm 1.4) \times 10^{10} \\ (4.2 \pm 0.5) \times 10^{10} \\ (4.3 \pm 2.2) \times 10^{10} \\ (3.7 \pm 1.6) \times 10^{10} \end{array}$

*Note.* For this particular study, there were means of 560 physical particles and 15 latex spheres per electron micrograph.

below). For example, it was possible that structures resembling knobs might be observed even when there was no gp120 present, i.e., false positives. On the other hand, any attempts to purify the virus would have caused additional loss of knobs.

# Quantifying gp120 and p24 losses by ELISA

To further examine the kinetics of viral decay, an HIV-1HXB3 stock was harvested after 48 hr of exponential growth (Fig. 1) and incubated at 37° as previously described (Layne *et al.*, 1991). At regular intervals, prespin, supernatant, and pellet samples were prepared (see Materials and Methods). To quantify the concentrations of envelope and core proteins, gp120 and p24 ELISAs were performed in parallel. Results from these ELISAs are shown in Figs. 4 and 5, respectively.

The concentration of viral-associated gp120 in the pellets (O in Fig. 4A) decreased with increasing preincubation time and reached a baseline level (O\*) by 168



Fig. 3. Determining the gp120 shedding rate by electron microscopy. The number of gp120 knobs on viral particles was evaluated after preincubation of a viral stock for 0, 12, and 60 hr at 37°. The mean number of knobs and standard deviation were calculated at each time point. The half-life (solid line) was an unweighted leastsquares fit to the data; 95% confidence limits (dotted lines) were calculated by a bootstrap algorithm. Error bars show  $\pm 1$  SD.

hr. A least-squares fit to the data (from 0 to 48 hr, lower solid line) yielded a half-life of 40 hr and 95% confidence limits of 35 to 45 hr. In conjunction, the concentration of soluble gp120 in the supernatant ( $\Delta$  in Fig.

4B) increased with time. Adding the gp120 concentrations in the pellet and supernatant together gave a total ( in Fig. 4A) that was constant during the entire experiment and matched the gp120 concentration in the prespin samples (I in Fig. 4B). At very long times, all the gp120 was lost from the pellet and a small background remained. A variety of phenomena could explain this result. For example, gp120 could be present on nonviral material, such as fragments of cell membranes (micelles). Alternatively, there could be subpopulations of viruses or knobs that did not shed. In either event, after the average gp120 concentration in the pellet at 168 hr (O\* in Fig. 4A) was subtracted from the data at earlier times, results that were disentangled from the background were obtained (
 in Fig. 4A). A least-squares fit to the data (from 0 to 48 hr, dotted line) yielded a half-life of 30 hr and 95% confidence limits of 27 to 33 hr. This corrected gp120 shedding rate agreed with direct EM measurements, to within a factor of 2, and agreed with gp120 ELISA measurements from two other decay experiments (summarized in Table 5).

The concentration of viral-associated p24 in the pellets (O in Fig. 5A) remained constant with preincubation time. In spite of this, the concentration of soluble p24 in the supernatant ( $\Delta$  in Fig. 5A) increased approximately twofold with time. This discrepancy in the data resides within experimental uncertainty. Alternatively, it could indicate that a small fraction (~20%) of viral particles "dissolved" over the 48-hr interval. Adding



Fig. 4. gp120 ELISA data from an HIV-1HXB3 stock that was preincubated at 37°. Symbols denote prespin samples ( $\blacksquare$ ), postspin supernatants ( $\Delta$ ), viral pellets (O), corrected viral pellets ( $\bullet$ ), and summed data ( $\square$ ). Results are the mean of six replicate wells per time point. Half-lives (solid lines) are unweighted least-squares fits to the data. The 95% confidence limits (reported with the half-life) were calculated by a bootstrap algorithm. Error bars show ±1 SD.

Log10 (p24) g / mi supernatant -6.5 -6.5 -7.0 -7.0 10 20 30 0 40 50 0 10 20 30 40 50 Preincubation Time (hrs) Preincubation Time (hrs)

Fig. 5. p24 ELISA data from an HIV-1HXB3 stock that was preincubation at 37°. Symbols denote prespin samples (III), postspin supernatants ( $\Delta$ ), viral pellets (O), and summed data ( $\Box$ ). Results are the mean of four replicate wells per time point. Half-lives (solid lines) were unweighted least-squares fits to the data; 95% confidence limits (reported with the half-life) were calculated by a bootstrap algorithm. Error bars show ±1 SD.

the p24 concentrations in the pellet and supernatant together gave a total (
in Fig. 5A) that was constant with time and matched the p24 concentrations in prespin samples (I in Fig. 5B). These p24 core protein data were consistent with electron microscope particle counts (Table 2), indicating that the envelope and core of HIV were relatively stable. The results also demonstrated that p24 was lost from viral pellets at a much slower rate than gp120 was shed. Therefore, the dissolution of intact viral particles was an insignificant factor contributing to the rate of gp120 shedding (O and ● in Fig. 4A).

#### Quantifying RNA polymerase loss at 37°

Reverse transcription is a necessary step in the life cycle of HIV (Goff, 1990). Quantitative RNA polymerase-activity assays were performed on the prespin, postspin, and pellet samples, with the goal of determining whether loss of polymerase activity influenced HIV infectivity. As shown in Fig. 6A, the activity of viralassociated RNA polymerase in the pellets (O) decreased with increasing preincubation time. A leastsquares fit to the data (upper line) yielded a half-life of 40 hr and 95% confidence limits of 35 to 45 hr. In addition, the activity of soluble polymerase in the supernatant ( $\Delta$  in Fig. 6B) decreased with time. A leastsquares fit to these data (lower line) yielded a half-life of 90 hr and 95% confidence limits of 30 to 150 hr. Adding the polymerase activities in the pellet and supernatant together gave a total ( in Fig. 6B) that also de-

creased during the experiment. The half-life of the sum was 40 hr, with 95% confidence limits of 36 to 44 hr. To within experimental uncertainty, the summed data matched the total polymerase activity in the prespin samples (I in Fig. 6B). The half-life of the prespin sample was 100 hr, with 95% confidence limits of 30 to 170 hr. These results demonstrated that the loss of viralassociated RNA polymerase activity was exponential and that the time constant was comparable to that of gp120 shedding. Similar results were obtained for another HIV-1HXB3 stock (data not shown).

В

#### Spontaneous viral inactivation at 37°

To correlate measurements of gp120 envelope shedding, p24 core dissolution, and RNA polymerase loss with the spontaneous inactivation of HIV, a series of quantitative infectivity assays were performed on preincubated HIV-1HXB3 stock. At each time point, the assays were conducted with CEM-SS and PHAstimulated H-PBMC target cells, both in the presence and in the absence of 0.5 nM sCD4 in the media. As shown in Fig. 7, this amounted to four different assays at each time point.

Spontaneous multihit inactivation of HIV was observed with both CEM-SS (Fig. 7A) and H-PBMC (Fig. 7B) target cells. At the start of the experiment, both target cell types scored a comparable number of SFU  $(216 \pm 7 \text{ for CEM-SS and } 241 \pm 8 \text{ for H-PBMC})$ . After 24 hr at 37°, the magnitude of viral inactivation was also similar (approximately 100-fold) for the four differ-





Fig. 6. RNA polymerase activity from an HIV-1HXB3 stock that was preincubated at 37°. Symbols denote prespin samples ( $\blacksquare$ ), postspin supernatants ( $\Delta$ ), viral pellets (O), and summed data ( $\Box$ ). Results are the mean of four replicate wells per time point. Half-lives (solid lines) were unweighted least-squares fits to the data; 95% confidence limits (reported with the half-life) were calculated by a bootstrap algorithm. Error bars show  $\pm 1$  SD.

ent assays. As shown in Figs. 7A and 7B, the profile of inactivation for both target cell types was threephased. There was an initial slow phase (less than 7 hr), followed by a fast phase (from 10 to 20 hr), and then another slow phase (greater than 22 hr). The processes responsible for generating this sigmoidal inactivation profile are considered under Discussion. Also, as shown in Figs. 7C and 7D, the blocking activity of sCD4 with CEM-SS and H-PBMC target cells was comparable in its overall profile. During the fast phase of viral inactivation (from 10 to 18 hr), sCD4 blocking activity increased significantly above baseline levels.

In spite of these similarities, there were still some observable differences in viral inactivation and sCD4 blocking. The duration of the initial slow phase of inactivation (plateau) was shorter for assays with CEM-SS cells ( $\sim$ 6 hr) than for assays with H-PBMC ( $\sim$ 9 hr). Preincubation times required for the 100-fold drop in infectivity were twofold shorter for CEM-SS cells (~18 hr) than for H-PBMC (~36 hr). For CEM-SS assays, the initial sCD4 blocking activity (0 to 10 hr in Fig. 7C) corresponded to the gp120-sCD4 Kassoc from chemical measurements (dotted line). For H-PBMC assays, however, the initial sCD4 blocking activity was nearly absent, falling far below the expected chemical  $K_{assoc}$ (dotted line). At longer preincubation times, the excursions in sCD4 blocking activity were more pronounced for CEM-SS assays than for H-PBMC assays. In other words, compared to CEM-SS cells, H-PBMC were more difficult to protect with sCD4 in the media. These results indicated that CEM-SS assays were unsaturated at a target cell density of  $1.4 \times 10^4$  ml<sup>-1</sup>, whereas the H-PBMC assays appeared partially saturated at this same target cell density. These observable differences in spontaneous viral inactivation (Figs. 7A and 7B) and sCD4 blocking (Figs. 7C and 7D) were consistent with the hypothesis that, compared to CEM-SS cells, HIV-1HXB3 required a smaller minimal number of unblocked gp120 molecules for efficient infection of H-PBMC. The rationale for this notion is considered under Discussion.

#### sCD4 blocking activity with viral preincubation at 37°

To further study how sCD4 blocking activity depended on spontaneous viral inactivation, a series of quantitative infectivity assays were performed with preincubated HIV-1HXB3 stock. In these assays, emphasis was placed on a larger number of sCD4 concentrations rather than on many sequential time points. To generate comparable data, assays were conducted with CEM-SS and H-PBMC target cells, under the same conditions as those in Fig. 7. As shown in Figs. 8A and 8B, this amounted to seven different assays at each of the three time points.

For CEM-SS assays without preincubation (O in Fig. 8A), the initial slope was linear and yielded a biological blocking activity of  $(1.5 \pm 0.2) \times 10^9 M^{-1}$ . At sCD4 concentrations greater than 0.75 n*M*, there was a positive synergy in blocking. For assays with 8 hr of preincubation ( $\Box$ ), the initial slope was linear and yielded a slightly increased blocking activity of  $(1.8 \pm 0.2) \times 10^9$ 



Fig. 7. The spontaneous inactivation of HIV-1HXB3 at 37°. At 1- to 2-hr intervals, the number of SFU in the viral stock was quantified with (A) CEM-SS and (B) H-PBMC target cells. For both target cell types, assays were conducted without (O) and with 0.5 nM sCD4 ( $\bullet$ ) in the media. Results are a mean of eight replicate wells per time point. Error bars show ±1 SD. The half-lives of spontaneous inactivation (solid lines) were unweighted least-squares fits to the data (from 0 to 6 hr) for assays without sCD4. The 95% confidence limits were calculated by a bootstrap algorithm. The ratios between the assays without and those with 0.5 nM sCD4 in the media are shown for (C) CEM-SS and (D) H-PBMC target cells. The ratios (normalized SFU) were calculated from data at each time point. Error bars show ±1 SD in these ratios. Dotted lines correspond to the expected amount of blocking for 0.5 nM sCD4 and a gp120-sCD4  $K_{assoc}$  equaling 1.5 × 10<sup>9</sup>  $M^{-1}$ .

 $M^{-1}$ . At the 1.25 and 1.5 n*M* sCD4, there were observable increases in the positive synergy. For assays with 16 hr of preincubation ( $\Delta$ ), the initial slope was again linear but yielded a fourfold increase in blocking activity, (5.8 ± 0.7) × 10<sup>9</sup>  $M^{-1}$ . At the 1.0 and 1.25 n*M* sCD4, there were correspondingly large increases in the positive synergy and at 1.5 n*M* sCD4, SFU were completely abolished. In the assays without preincubation (O), sCD4 blocking activity corresponded to the gp120–sCD4  $K_{assoc}$  from chemical measurements

(Layne *et al.*, 1990). The mechanism for the increasing positive synergy is considered in the Discussion.

For H-PBMC assays without preincubation (O in Fig. 8B), the linear slope yielded a biological blocking activity of  $(2.4 \pm 0.9) \times 10^8 M^{-1}$ , indicating partial saturation. For assays with 8 hr of preincubation ( $\Box$ ), the linear slope yielded an eightfold increase in blocking activity,  $(2.0 \pm 0.2) \times 10^9 M^{-1}$ . This number corresponded to the gp120–sCD4  $K_{\rm assoc}$  from chemical measurements (Layne *et al.*, 1990). For assays with 16 hr of



FIG. 8. Biological blocking activities of sCD4 (solid lines) were determined after preincubation of an HIV-1HXB3 stock at 37°. (A) For CEM-SS target cells, sCD4 blocking activities were  $(1.5 \pm 0.2) \times 10^9$ ,  $(1.8 \pm 0.2) \times 10^9$ , and  $(5.8 \pm 0.7) \times 10^9 M^{-1}$  at 0 (O), 8 (D), and 16 ( $\Delta$ ) hr, respectively. (B) For H-PBMC target cells, sCD4 blocking activities were  $(2.4 \pm 0.9) \times 10^9$ ,  $(2.0 \pm 0.2) \times 10^9$ , and  $(1.1 \pm 0.1) \times 10^9 M^{-1}$  at 0 (O), 8 (D), and 16 ( $\Delta$ ) hr, respectively. For CEM-SS assays, blocking activities were weighted least-squares fits to the four lower sCD4 concentrations. For H-PBMC assays blocking activities were weighted least-squares fits to the four lower sCD4 concentrations. For H-PBMC assays blocking activities were weighted least-squares fits to the four lower sCD4 concentrations. For H-PBMC assays blocking activities were weighted least-squares fits to all the data. Results are the mean of eight replicate wells per data point. Error bars show ±1 SD. The dotted lines were drawn to clarify trends in the data.

preincubation ( $\Delta$ ), the linear slope yielded a twofold decrease in blocking activity,  $(1.1 \pm 0.1) \times 10^9 M^{-1}$ . The increasing-and-decreasing slopes with preincubation time (Fig. 8B) were similar to the up-and-down profile of sCD4 blocking (Fig. 7D). However, in contrast to the CEM-SS assays (Fig. 8A), the H-PBMC assays showed no evidence of positive synergy (Fig. 8B). The explanation of these results is considered under Discussion.

# Phosphonoformate inhibitory activity with viral preincubation at 37°

The shedding of gp120 envelope proteins (Figs. 3 and 4) and the loss of RNA polymerase activity (Fig. 6) occurred at rates that resembled the initial rate of HIV inactivation (from 0 to 6 hr in Figs. 7A and 7B). Since these rates were at least threefold faster than the dissolution of p24 core proteins (Fig. 5), it appeared likely that either one or both of these processes governed initial HIV decay. The observation that sCD4 blocking varied with viral preincubation (Fig. 8) has already implicated gp120 shedding in HIV inactivation (Layne *et al.*, 1991). It thus remained to be settled how the loss of virion-associated RNA polymerase activity contributed to spontaneous viral decay.

To make this assessment, a series of RNA polymerase inhibition assays were performed. As explained under Materials and Methods and shown in Fig. 9, this amounted to four assays at 0 (O), 8 ( $\Box$ ), 16 ( $\triangle$ ), and 24 (◊) hr of preincubation, plus three side-effect assays (•). When phosphonoformate was added after the target cells were infected and washed, there was no observable inhibition of infectious events (• in Fig. 9). Thus, the presence of this antiviral agent in microtiter wells did not alter the number of SFU. For assays with phosphonoformate in the media during infection, there were observable inhibitions in SFU compared to the relevant controls (Fig. 9). For assays with 25  $\mu M$  phosphonoformate, the inhibition of SFU ranged from 1.9to 2.8-fold. The slight differences in inhibitory activity among the four preincubation times, however, resided within experimental uncertainty and did not reveal any strong trends. For assays with 50  $\mu M$  phosphonoformate, on the other hand, the inhibition of SFU ranged from 1.9- to 5.9-fold and were correlated with preincubation time. For assays with 100  $\mu M$  phosphonoformate, the inhibition of SFU ranged from 15- to 34-fold and were strongly correlated with preincubation time.

The half-life of RNA polymerase activity in intact virions was 40  $\pm$  5 hr (Fig. 6). Thus, during the viral inactivation assays shown in Fig. 7, approximately half of the RNA polymerase activity was lost. Figure 9 shows that when half of the RNA polymerase activity was inhibited by 25  $\mu$ *M* phosphonoformate, there was no observable difference among the four preincubated HIV-1HXB3 stocks. Therefore, for preincubation times less than 40 hr, the results clearly demonstrated that spontaneous



Fig. 9. The graph shows the inhibitory action of phosphonoformate on an HIV-1HXB3 stock that was preincubated at 37°. Infectivity assays with CEM-SS target cells were conducted on stocks that were preincubated for (O) 0, ( $\Box$ ) 8, ( $\Delta$ ) 16, and ( $\Diamond$ ) 24 hr. At each preincubation time, the ordinates (normalized SFU) were calculated by dividing the assays without phosphonoformate by the assays with phosphonoformate. To evaluate the side effects of phosphonoformate on syncytium formation, three additional assays were conducted without viral preincubation and without phosphonoformate in the media. Subsequently, phosphonoformate was added to the media after infecting, washing, and plating of the target cells. Normalizing these three assays by the relevant control assay without phosphonoformate (O) yielded ordinates ranging from to 0.99 and 1.03 (•). This result demonstrated that phosphonoformate did not inhibit the formation of syncytia in the microtiter wells. For all assays, results are the mean of eight replicate wells per data point. Error bars show  $\pm 1$  SD

HIV inactivation was independent of RNA polymerase activity. In other words, virions contained a surplus of active RNA polymerase. For much longer preincubation times, however, the loss of RNA polymerase activity appears to contribute to HIV inactivation (see Discussion).

#### Calculating the protein content per virion

Data from the foregoing physical, chemical, and biological assays permitted direct calculation of the number of p24 molecules per virion, gp120 molecules per virion, and active RNA polymerase molecules per virion. These results are summarized in Table 3.

Between 0 and 48 hr of preincubation, p24 concentrations in the viral pellets were conserved (O in Fig. 5A). Averaging all the data (40 ELISA determinations) gave the p24 concentration reported in Table 3. Using the average virus particle concentrations from Table 2 yielded a mean of  $5 \times 10^{-17}$  g of p24 core protein per

particle. Converting this number with a molecular weight of  $2.4 \times 10^4$  g mol<sup>-1</sup> yielded a mean of 1200 p24 molecules per virion. Similar results were also obtained for two independent HIV-1HXB3 stocks (data not shown).

Between 0 and 48 hr of preincubation, gp120 concentrations in the viral pellets (O and • in Fig. 4A) declined at a rate that was exponential. Table 3 shows the gp120 concentrations in the viral pellets at 0 and 48 hr of preincubation, with the background gp120 concentration at 168 hr subtracted (O\* in Fig. 4A). Using the average virus particle concentrations from Table 2 yielded means of  $2 \times 10^{-18}$  and  $7 \times 10^{-19}$  g of gp120 envelope protein per particle at 0 and 48 hr, respectively. Converting these numbers with a molecular weight of  $1.5 \times 10^5$  g mol<sup>-1</sup> yielded a mean of 10 and 4 gp120 molecules per physical particle, respectively. Electron microscopy revealed that approximately 90% of the physical particles were devoid of knobs (data not shown). Thus, the average number of gp120 molecules on infectious virions may be as much as 10-fold larger (see Discussion). Similar results were also obtained for another independent HIV-1HXB3 stock (data not shown).

Between 0 and 24 hr of preincubation, RNA polymerase activity in the viral pellets (O in Fig. 6A) declined at an exponential rate. Table 3 shows the RNA polymerase concentration in the viral pellets at 0 and 24 hr of preincubation. Using the average virion density from Table 2 yielded means of  $1.6 \times 10^{-17}$  and  $1.1 \times 10^{-17}$  g of active RNA polymerase per particle at 0 and 48 hr, respectively. Converting this number with a molecular weight of  $1.17 \times 10^5$  g mol<sup>-1</sup> yielded means of 80 and 55 active molecules per virion, respectively. Similar results were also obtained for duplicate assays on the same HIV-1HXB3 stock (data not shown).

#### Calculating infectious to noninfectious virion ratios

For viral stocks without preincubation (at 0 hr in Figs. 7A and 7B), quantitative infectivity assays with CEM-SS and H-PBMC target cells detected 216  $\pm$  7 and 241  $\pm$  8 SFU per microtiter well, respectively. To convert the number SFU per well to the number SFU per milliliter of viral stock, a multiplicative factor of 55.6 was used. These converted data are shown in the first two rows in Table 4. Next, dividing this number by the average virion density (from Table 2) yielded a SFU to physical particle ratio of approximately  $3 \times 10^{-7}$  for both target cells types. That is, about one in three million particles were infectious.

Table 4 shows converted data for quantitative infectivity assays with three other HIV-1HXB3 stocks. For all stocks, the ratio of infectious to noninfectious virion

PROTEIN CONTENT OF HIV-THAD3 PARTICLES						
Preincubation time (hr)	Protein in viral pellet (g ml <sup>-1</sup> )	Virion density (ml <sup>-1</sup> )	Protein content per virion (g)	No. of molecules per virion		
Core p24						
0-48 average	$(1.6 \pm 0.2) \times 10^{-6}$	$(3.7 \pm 1.6) \times 10^{10}$	$(4.4 \pm 2.6) \times 10^{-17}$	1200 ± 700		
Envelope gp120						
0	$(6.9 \pm 0.4) \times 10^{-8}$	$(3.7 \pm 1.6) \times 10^{10}$	$(1.9 \pm 0.9) \times 10^{-18}$	$10 \pm 5$		
48	$(2.7 \pm 0.2) \times 10^{-8}$	$(3.7 \pm 1.6) \times 10^{10}$	$(7.4 \pm 3.8) \times 10^{-19}$	4 ± 2		
RNA polymerase						
0	$(6.1 \pm 0.3) \times 10^{-7}$	$(3.7 \pm 1.6) \times 10^{10}$	$(1.6 \pm 0.8) \times 10^{-17}$	80 ± 40		
24	$(4.1 \pm 0.1) \times 10^{-7}$	$(3.7 \pm 1.6) \times 10^{10}$	$(1.1 \pm 0.5) \times 10^{-17}$	55 ± 27		

 TABLE 3

 PROTEIN CONTENT OF HIV-1HXB3 PARTICLES

Note. All data are reported as the mean  $\pm$  1 SD.

ranged from a low of  $3 \times 10^{-7}$  to a high of  $1 \times 10^{-4}$ . These results demonstrated that ratios depended on target cell density. Lower target cell densities (unsaturated assays) were associated with smaller ratios, and higher target cell densities (saturated assays) were associated with larger ones (Layne *et al.*, 1989).

#### Comparing the results from three decay assays

Table 5 summarizes the results of gp120 ELISA, p24 ELISA, and RNA polymerase activity assays for three separate HIV-1HXB3 stocks. Stock 1 was already examined in detail (see Figs. 4–6). Stocks 2 and 3 provide useful comparisons for evaluating the reproducibility and uncertainty in the data. The results from all three stock demonstrate that the total amounts (prespin and summed data) of p24 and gp120 protein in the assays were conserved with time. The half-lives for gp120 shedding in the viral pellets were remarkable similar (mean of 28 hr) among all three stocks. The results also demonstrate that the p24 half-lives in the viral pellets

were three- to fourfold larger than the half-lives of gp120 and RNA polymerase activity.

### DISCUSSION

In this study, a variety of physical, chemical, and biological data on HIV-1 stocks growing exponentially in transformed cell cultures were collected. This permitted correlations between different types of data and determinations of the number of gp120 envelope, p24 core, and RNA polymerase molecules per virion. To our knowledge, such investigations have not been previously reported for HIV, nor for other related lentiviruses and retroviruses.

Direct measurements of the envelope shedding rate by electron microscopy (Fig. 3) and gp120 ELISA (Fig. 4) were comparable to within a factor of 2. In conjunction, we found that the loss of virion-associated RNA polymerase activity (Fig. 6) took place at rate similar to the rate of gp120 shedding. A twofold diminution of RNA polymerase activity by 25  $\mu$ M phosphonoformate,

Target cell type	Target cell density (ml <sup>-1</sup> )	SFU (ml <sup>-1</sup> ) (mean ± SD)	Virion density (mI <sup>-1</sup> ) (mean ± SD)	SFU to virion ratio (mean ± SD)
CEM-SS	$1.4 \times 10^{4}$	$(1.2 \pm 0.04) \times 10^4$	$(3.7 \pm 1.6) \times 10^{10}$	$(3.2 \pm 1.5) \times 10^{-7}$
H-PBMC	$1.4 \times 10^{4}$	$(1.3 \pm 0.04) \times 10^4$		$(3.6 \pm 1.7) \times 10^{-7}$
CEM-SS	$1.6 \times 10^{5}$	$(1.5 \pm 0.04) \times 10^{5}$	$(4.9 \pm 2.3) \times 10^9$	$(3.0 \pm 1.5) \times 10^{-6}$
CEM-SS	$4.0 \times 10^{6}$	$(1.9 \pm 0.07) \times 10^{6}$		$(3.9 \pm 1.9) \times 10^{-4}$
CEM-SS	$5.0 imes10^5$	$(8.5 \pm 0.4) \times 10^4$	$(2.6 \pm 0.8) \times 10^9$	$(3.3 \pm 1.2) \times 10^{-6}$
CEM-SS	$2.0 imes10^6$	$(2.6 \pm 0.1) \times 10^5$		$(1.0 \pm 0.4) \times 10^{-4}$
CEM-SS	$2.0 \times 10^{6}$	$(5.0 \pm 0.24) \times 10^{5}$	$(1.2 \pm 0.6) \times 10^{9}$	$(4.1 \pm 2.2) \times 10^{-4}$

 TABLE 4

 Ratio of Infectious to Noninfectious Particles

Note. The table shows results for four separate HIV-1HXB3 stocks. All the stocks were assayed without preincubation. SFU were averaged from eight microtiter wells.

HALF-LIFE OF p24, gp120, and RNA POLYMERASE ACTIVITY									
	p24 core protein			gp120 envelope protein		RNA polymerase activity			
Stock	Prespin	Summed	Pellet	Prespin	Summed	Pellet	Prespin	Summed	Pellet
No. 1 No. 2	>270 >170	No loss 430 ± 60	No loss 100 ± 20	>280 No loss	>1000 >500	$30 \pm 3$ $25 \pm 5$	100 ± 70	40 ± 4 	40 ± 5
NO. 3	NO IOSS	INO IOSS	>110	>240	$60 \pm 10$	29 ± 5			

ТΑ	В	L	Е	5
	-	-	-	-

Note. Summed data are the postspin supernatant and pellet data added together. No loss corresponds to least-squares fit to the data having either zero or positive slope with preincubation time. All data are reported as the mean  $\pm 2$  SD. Half-life measured in hours.

however, did not distinguish the infectivity of viral stocks with 24 hr preincubation from those without preincubation (Fig. 9). Thus, the loss of RNA polymerase activity did not contribute significantly to HIV inactivation at short preincubation times. In addition, we found the lipid envelope covering virions (Table 2) and p24 core proteins within virions (Fig. 5) were stable compared to shedding and loss of RNA polymerase activity. This demonstrated that the physical breakup of viral particles did not contribute significantly to the rates of envelope shedding and polymerase loss. When considered together, the data demonstrate that the initial loss of HIV-1 infectivity was most closely correlated with the spontaneous shedding of gp120 (Gelderblom et al., 1985; McKeating et al., 1991). The kinetics of HIV inactivation at longer preincubation times is considered below.

For independent and equivalent gp120 molecules, mathematical models of HIV infection predicted that plots of normalized SFU versus sCD4 concentration should yield a straight line (Layne et al., 1989). When the data for such plots came from unsaturated infectivity assays, the initial slopes were both expected and found to equal the gp120-sCD4 Kassoc from chemical measurements (Layne et al., 1990). Upward curvature in normalized SFU plots indicated blocking greater than proportional to gp120-sCD4 complex formation (positive synergy). Conversely, downward curvature indicated blocking less than proportional to complex formation (negative synergy). In Fig. 8A, the initial linear slopes (at preincubation times of 0 and 8 hr) were similar to the sCD4-gp120 Kassoc from chemical measurements. For sCD4 concentrations less than 0.75 nM, this result demonstrated that sCD4 blocking activity was proportional to complex formation and verified that the viral infectivity assays were indeed unsaturated. At higher concentrations, however, the positive synergy and its increase with preincubation time support the hypothesis that HIV required a minimal number of unblocked gp120 molecules for efficient infection of CD4<sup>+</sup> cells. The increasing positive synergy appeared

with time because gp120 shedding acted as a surrogate for sCD4 blocking (Layne *et al.*, 1991).

For unsaturated infectivity assays, mathematical models predicted that the initial slope of a viral decay plot should correspond to the combined rates of gp120 shedding and particle dissolution (Layne et al., 1989). In agreement with this, the initial rates of viral decay (0 to 6 hr in Figs. 7A and 7B) were similar to the combined rates to within a factor of 2. After this initial phase, however, the multihit inactivation rate was much faster than expected from simple assumptions of independent and equivalent gp120 molecules. This finding further corroborated the minimal number hypothesis, because virions falling below a threshold would accentuate the observed rate of multihit inactivation. During the final phase of viral decay (preincubation times greater than 20 hr), there was a slowing in the rate for both types of target cells. This slowing was consistent with the single-hit inactivation of virions that were far below the minimal number threshold. In this case, the inactivation rate of these minimally infectious particles should equal the combined rates of gp120 shedding, particle dissolution, and loss or RNA polymerase activity. The data in Figs. 7A and 7B were certainly consistent with this prediction, but their large uncertainties did not permit reliable calculations of halflives and confidence limits.

As HIV infectivity decreased with time, there was a peculiar up-and-down profile in the blocking activity of sCD4 (Figs. 7C and 7D). The upward portion of this profile was analogous to an increasing positive synergy with preincubation time (Fig. 8A). To understand the downward portion, however, we must consider how sCD4 blocking was affected by the distribution of gp120 molecules in the viral population. At short preincubation times, there was a "homogeneous" population of virions with more than the minimal number of free gp120 molecules. The small amounts of sCD4 in the infectivity assays (• in Figs. 7A and 7B) did not push the population below threshold. Thus, relative to the infectivity assays without sCD4 (O in Figs. 7A and

7B), blocking was proportional to gp120-sCD4 complex formation. At intermediate preincubation times, there was a "heterogeneous" population of virions that were both above and below the minimal number. In this case, sCD4 pushed a larger fraction of the population below threshold. Thus, relative to infectivity assays without sCD4, blocking was greater than proportional to complex formation. At long preincubation times, there was a homogeneous population of virions with less than the minimal number of free gp120 molecules. The sCD4 in the assays acted on a viral population that was already below threshold. Thus, relative to infectivity assays without sCD4, blocking was again proportional to complex formation. By this reasoning, sCD4 blocking activity would be expected to subside with preincubation times. As shown in Fig. 8B, the sCD4 blocking activity indeed declined by twofold between preincubation times of 8 and 16 hr.

At long preincubation times (Fig. 7), both the reduced rate of HIV decay and the up-and-down blocking profile were most consistent with the notion of minimally infectious particles that were below threshold. For completeness, however, two alternative explanations for these data were considered. The first was that there was a subfraction of virions (about 10<sup>-2</sup> of the infectious population) that were highly resistant to blocking by sCD4. This resistant population became evident only after the disappearance of the more labile and susceptible population. Since higher sCD4 concentrations (5 nM) blocked infection by more than 500fold (data not shown), this explanation seemed unlikely. The second was that there was a subfraction of target cells (about 10<sup>-2</sup> of the total population) that were highly susceptible to infection. Since the overall magnitude of HIV inactivation was similar for both CEM-SS and H-PBMC target cells, however, this latter explanation also seemed unlikely.

At short and long preincubation times, both CEM-SS and H-PBMC target cells detected similar numbers of SFU (Figs. 7A and 7B). Also both cell types had sigmoidal profiles of HIV decay, reflecting the influence of a threshold for efficient infection. There were, nevertheless, several noteworthy differences in these infectivity assays. First, the time interval in which the 100-fold loss of viral infectivity took place was nearly twofold longer for H-PBMC ( $\sim$  36 hr) than for CEM-SS ( $\sim$  18 hr) target cells. For the H-PBMC assays, the initial phase of slow inactivation was several hours longer and the intermediate multihit phase had a slower rate. Second, for identical cell densities and assay conditions, the blocking activity of sCD4 was less for H-PBMC target cells than for CEM-SS target cells (Figs. 7C and 7D). For H-PBMC assays, the initial sCD4 blocking activity was below the gp120-sCD4 K<sub>assoc</sub> (dotted line), indicating the effects of saturation (Layne et al., 1991). For CEM-SS assays, the initial sCD4 blocking activity equaled K<sub>assoc</sub>, indicating unsaturated conditions. Third, the up-and-down profile of sCD4-blocking activity was less pronounced for assays with H-PBMC target cells than for assays with CEM-SS target cells. All these seemingly incongruent observations are consistent with the ideas that HIV-1HXB3 required a smaller minimal number of gp120 molecules for efficient infection of H-PBMC than for infection of CEM-SS target cells. This follows because the effects of multihit inactivation are directly related to the minimal number. Smaller thresholds have less pronounced effects on viral decay and vice versa. Also the effects of assay saturation are inversely proportional to the minimal number. Thus, at a particular density, target cells with a smaller threshold are more likely to underestimate the gp120-sCD4 K<sub>assoc</sub>. The results in Fig. 8 provide further support for these ideas. Compared to assays with CEM-SS target cells (Fig. 8A), assays with H-PBMC target cells (Fig. 8B) yielded lower sCD4 blocking activities with preincubation time and showed no signs of positive synergy with increasing sCD4 concentration.

The surfaces of both HIV and CD4<sup>+</sup> cells are studded with adhesion molecules and covered by a diffuse coat of sugar molecules (glycocalyx) that are negatively charged (Fenouillet et al., 1989; Springer, 1990). This similar feature suggests that the initial binding of HIV to a target cell is, in many ways, parallel to the adhesive interaction between two immune cells. For such cell-cell interactions, it was shown that adhesion between two surfaces represents a first-order thermodynamic phase transition which takes place only when a certain threshold number of interactions between adhesion molecules is exceeded (Bell et al., 1984; Dembo and Bell, 1987). If the density of adhesion molecules is below this critical number, the cellular surfaces will simply not adhere (Plunkett et al., 1987). The exact value of the critical number is a function of several variables, most importantly the strength of the repulsive electrostatic potential between the surfaces and the association constant of the adhesion molecules for their complementary receptors. This viewpoint suggests that the critical number of gp120 molecules for efficient infection arises from repulsive forces between viral and cellular glycocalyces and attractive ones between gp120 and CD4. In order to overcome the protective barrier enshrouding the CD4<sup>+</sup> cells, a critical number of gp120-CD4 interactions must form. Once the amount of gp120 falls below the critical number, a virion may still penetrate a cell (for example, by colliding with cells or at regions on cells having thinner barriers) but the odds will be slim. For the H-PBMC and

CEM-SS assays (Figs. 7 and 8), the observed differences in the sigmoidal profile of HIV decay and sCD4 blocking appear to be caused by smaller and larger cell–surface barriers, respectively. This view suggests that a variety of cell–surface interactions are important factors in infectivity (Layne and Dembo, 1992; Lu *et al.*, 1992).

From this study, there were sufficient data for estimating the minimal number of gp120 molecules for efficient infection of CEM-SS target cells. When more than 0.75 nM sCD4 was added to infectivity assays with CEM-SS target cells, there was a positive synergy (Fig. 8). Using a gp120–sCD4  $K_{\text{assoc}}$  of  $1.5 \times 10^9 M^{-1}$ and the sCD4 concentration at which upward curvature began permitted us to directly estimate the minimal fraction of unblocked gp120 molecules. For equilibrium blocking conditions (Layne et al., 1990), this estimate gave a free gp120 fraction of  $[1 + (1.5 \times 10^9 M^{-1})]$  $\times (7.5 \times 10^{-10} M)]^{-1} \approx 0.47$ , or approximately 50% for the minimal fraction. At zero preincubation time, gp120 ELISA detected a mean of  $10 \pm 5$  gp120 molecules per viral particle (Table 4). Since electron microscopy revealed that approximately 90% of all viral particles were devoid of knobs (data not shown), this mean must be multiplied by a factor of 10 to normalize the data. Thus, for the subpopulation of particles with knobs, there were as many as  $100 \pm 50$  gp120 molecules per viral particle. Multiplying the normalized data by the minimal fraction of 50% yields a minimal number of 50 ± 25 gp120 molecules. Since HIV-1 can have up to 72 knobs on its surface (Özel et al., 1988) and since these knobs are either trimers or tetramers (Gelderblom et al., 1988; Schawaller et al., 1989; Earl et al., 1990), we estimate a maximum of 210 to 280 gp120 molecules per virion. Therefore, compared to HIV-1HXB3 particles with all their knobs, we estimated a minimal fraction of 10 to 30% for CEM-SS cells. Since H-PBMC showed no signs of positive synergy at the highest sCD4 concentrations (1.5 nM) used in this study, it appeared that the minimal number of knobs for these "physiologic" target cells was smaller.

For viral stocks without preincubation, we found that there were  $80 \pm 40$  active RNA polymerase molecules per virion (Table 3). This result was based on the enzymatic activity of a known amount of recombinant RNA polymerase from HIV-1IIIB, which closely resembles the HIV-1HXB3 clone. The recombinant enzyme was a highly purified p66/p51 heterodimer, resembling the native enzyme found in virions (Veronese *et al.*, 1986; Furman *et al.*, 1991). For other retroviruses, such as Moloney murine leukemia virus (Panet *et al.*, 1978), Rauscher mouse leukemia virus (Krakower *et al.*, 1977), and avian myleoblastosis virus (Panet *et al.*, 1975; Bauer *et al.*, 1980; Kacian *et al.*, 1971), the RNA polymerase contents were found to range between 17 and 110 molecules per virion. Thus, the number of polymerase molecules per HIV particle was similar to that for avian and murine type C retroviruses.

To our knowledge, the half-life of viral-associated RNA polymerase activity at 37° has not been previously reported for HIV. Temperature-sensitive RNA polymerases have been characterized from Moloney leukemia virus (MLV) but they were selected from mutant viral strains rather than from a cloned laboratory isolate (Goff et al., 1981). For MLV, previous studies also found that RNA polymerase activity was highly labile at 44° and that the loss of RNA polymerase activity correlated with the spontaneous decay of infectivity at 44° (Gerwin and Levin, 1977). In addition, inhibiting the incorporation of RNA into virions by actinomycin D had no measurable effects on thermolability. It was thus concluded that the thermal properties of MLV RNA polymerase were independent of the viral genome. Since the loss of polymerase activity and its decay were investigated at elevated temperatures, however, the relevance of these MLV findings to the present ones must await further clarification. Other studies with HIV-1 have found that recombinant RNA polymerase was not autolytic in vitro (Mizrahi et al., 1989) and that recombinant protease did not degrade heterodimeric p66/p51 RNA polymerase in vitro (Meek et al., 1989). Thus, for HIV-1, several plausible mechanisms for the loss of RNA polymerase activity have already been ruled out by the investigations of others. The mechanisms responsible for the spontaneous loss of RNA polymerase activity in virions remain undefined at present.

The phosphonoformate inhibition assays demonstrated that a twofold perturbation of RNA polymerase activity did not distinguish viral stocks with 24 hr preincubation from those without preincubation (25  $\mu M$  in Fig. 9). Thus, at short preincubation times, we found that virions possessed redundant amounts of RNA polymerase activity. At higher phosphonoformate concentrations (50 and 100  $\mu$ M), there were clear differences in the preincubated viral stocks. As shown in Fig. 9, the inhibitory activity of phosphonoformate was directly correlated with preincubation at 37°. This result suggested that the loss of RNA polymerase activity contributed to HIV decay at preincubation times greater than the polymerase half-life (about 40 hr in Fig. 6). This additional mechanism of HIV inactivation helps to account for the small ratio of infectious to noninfectious virions in viral stocks that were grown exponentially (Table 4). From the limited number of phosphonoformate inhibitions assays, though, we cannot tell whether HIV inactivation was proportional to the loss of virion-associated RNA polymerase activity or whether there was a threshold number of polymerase molecules for efficient infection. Further experiments will be required for clarifying this matter.

In this study, the ratios of infectious to noninfectious virions ranged from 10<sup>-4</sup> to 10<sup>-7</sup> and were correlated with the density of CD4<sup>+</sup> target cells in the infectivity assays (Table 4). At the lower cell densities, the ratios were smaller and vice versa. Four of the viral infectivity assays were carried out at CD4<sup>+</sup> cell densities that were 10- to 100-fold smaller than those of blood, where typical densities are around  $5 \times 10^6$  cells ml<sup>-1</sup>. These particular results (Table 4) may underestimate the infectious-to-noninfectious ratios that are characteristic of blood. In lymph and lymph nodes, typical CD4<sup>+</sup> cell densities are 10<sup>7</sup> to 10<sup>9</sup> cells ml<sup>-1</sup>, respectively. It therefore remains unclear whether a significantly larger fraction of the so-called "noninfectious" virions would infect at higher target cell densities. Recently, it was suggested that HIV burdens in lymphoid organs (i.e., the fraction of PCR-positive cells) were approximately 10-fold larger than the ones in blood (Pantaleo et al., 1991). With this finding, it is tempting to speculate that larger ratios of infectious-to-noninfectious virions in lymphoid organs may contribute to this elevated burden.

Thin-section immunoelectron microscopy (Gelderblom, 1987) and electron microscope tomography (Höglund et al., 1991) of HIV-1 indicated that a p24 shell covers the viral genome. The volume of this shell was approximately  $3 \times 10^7 \text{ Å}^3$ , which corresponds to a hollow cylinder with a length of 1200 Å, a diameter of 200 Å, and a thickness of 50 Å. Since proteins have a mean density of 1.23 D Å<sup>-3</sup> and 1 D =  $1.67 \times 10^{-24}$  g (Barrow, 1974), we estimated that an individual virion contains approximately  $4 \times 10^{-17}$  g of p24. This estimate resembles those of other investigators,  $\sim 10^{-16}$  g (Vogt, 1965; Luftig et al., 1990). In this study, it was found that HIV-1 contains approximately  $5 \times 10^{-17}$  g of p24 per virion (Table 3), which agrees closely with the above estimates. This measurement is based on p24 ELISA, which was calibrated against a recombinant p24 standard. Uncertainties in the concentration of this particular standard and differences in the capture and detection efficiency of recombinant and native viral proteins will contribute to errors in this measurement.

The remarkable stability of the p24 core (Table 5) and lipid membrane covering HIV-1 (Table 2) suggested that these data may have some use in ascertaining the stability of virions in the plasma of infected persons. In some cases, clinical studies found correlations between the viral titers and p24 concentrations in plasma (Ho *et al.*, 1989; Coombs *et al.*, 1989; Clark *et al.*, 1991; Daar *et al.*, 1991). In these instances, the viral titers ranged from 10<sup>1</sup> to 10<sup>4</sup> TCID<sub>50</sub> ml<sup>-1</sup> and the corresponding p24 concentrations in plasma ranged from 10 to 1000 pg ml<sup>-1</sup>. Based on  $5 \times 10^{-17}$  g of p24 per virion, these plasma p24 concentrations correspond to  $2 \times 10^5$  to  $2 \times 10^7$  virions ml<sup>-1</sup>, respectively. Thus, in the plasma of infected individuals, we estimate that the ratio of infectious to noninfectious virions varied from  $5 \times 10^{-5}$  to  $5 \times 10^{-4}$ . These ratios were comparable to the ones shown in Table 4, suggesting that human plasma does not contain surface-active factors with pronounced viricidal activities.

Among HIV researchers, there has been some debate on how to produce "optimal" viral stocks for use in antiviral screening assays and vaccine trials in animals. Resolving this debate is important because the physical state of viral particles can greatly influence the activities of antiviral agents that attack extracellular (Fig. 8) and intracellular (Fig. 9) targets. We believe that the close correlation between gp120 shedding (Fig. 4) and initial HIV decay (Fig. 7) provides a straightforward method for judging whether stocks are indeed optimal. To achieve this, two pieces of information are required. The first is a measure of the doubling time for viral stocks replicating exponentially in cell cultures. A good method for making such an estimate is illustrated in Fig. 1. The second is a measure of the half-life of gp120 shedding. Such a determination can come from physical measurements with electron microscopy (Fig. 3), from chemical measurements with ap120 ELISA (Fig. 4), or from unsaturated infectivity assays (see the initial slopes in Figs. 7A and 7B). From these data, it is simple to see whether the doubling time to half-life ratio is smaller or larger than one. If this ratio is smaller than one, then new virions are expressed at a rate that outpaces their spontaneous decay. Conversely, if this ratio is larger than one, then new virions are expressed at a rate that lags behind their spontaneous decay. From this perspective, it is easy to recognize that the mean number of gp120 molecules per virion will be larger in stocks with smaller growth-to-decay ratios. Therefore, to maximize the number gp120 molecules per virion, HIV stocks must be grown such that the doubling time is much smaller than the half-life for spontaneous shedding. Based on a gp120 half-life of 30 hr (Fig. 4), we calculate grow-to-decay ratios of 0.067 and 0.23 for the fastest and slowest HIV-1HXB3 stocks, respectively (Fig. 1). For growth times less than 48 hr, both viral stocks meet our criteria for optimization. In previous growth studies in vitro (Fenyö et al., 1989), HIV strains were categorized as rapid-high or slow-low. Because of its simplicity, this classification still has some utility for growth characteristics and tropism. We believe, however, that doubling times, half-lives, and growth-to-decay ratios provide a more complete classification of viral characteristics.

In this study, we found that the processes governing the decay of HIV infectivity were exponential. Whether this simple behavior is particular to HIV-1HXB3 or applicable to other viral strains and isolates must await future studies. For a number of wild-type strains, the relationships between the rates of particle lysis, p24 dissolution, gp120 shedding, RNA polymerase loss, and spontaneous HIV decay require determination. The minimal number of gp120 molecules for efficient infection of CD4<sup>+</sup> cells also requires determination. This number is particularly important because an inverse relationship between immunoglobulin blocking activity and the critical number is perceivable. Larger critical numbers will require correspondingly smaller humoral responses and vice versa. To determine whether this relationship influences the development of HIV vaccines with broad efficacy, it will be important to see how the critical number varies between divergent wildtype strains and CD4<sup>+</sup> cells from a number of individuals.

#### ACKNOWLEDGMENTS

We thank Drs. S. A. Stern (ERC BioServices) for Celltech recombinant gp120, R. Ward (Genentech) for recombinant sCD4, R. C. Gallo for HIV-1HXB3, and D. A. Katzenstein (Stanford University) for stimulating discussions. We also thank Ms. J. Condino (Beckman Instruments) for the references on sedimentation coefficients. Work by S.P.L. and M.D. was financially supported by the U.S. Army Medical Research and Development Command (DAMD17-90-MM0545) and U.S. Department of Energy. Work by J.L.S., M.J.M., S.R.C., and P.L.N. was supported by the National Institutes of Health. Work by J.P.M. was supported by the U.K. Medical Research Council AIDS Directed Program and was carried out at the Chester Beatty Laboratory, London. Work by H.R. and H.R.G. was supported by the Ministry for Research and Technology, Bonn, Germany (01 ZR 89018 to H.R.G.).

#### REFERENCES

- ANDERSON, N. G. (1968). Analytical techniques for cell fractions VIII. Analytical differential centrifugation in angle-head rotors. *Anal. Biochem.* 23, 72–83.
- BALTIMORE, D., and SMOLER, D. (1971). Primer requirements and template specificity of the DNA polymerase of RNA tumor viruses. *Proc. Natl. Acad. Sci. USA* **68**, 1507–1511.
- BARROW, G. M. (1974). "Physical Chemistry for the Life Sciences." McGraw-Hill, New York.
- BAUER, G., and TEMIN, H. M. (1980). Radioimmunological comparison of the DNA polymerases of avian retroviruses. J. Virol. 33, 1046–1057.
- BELL, G. I., DEMBO, M., and BONGRAND, P. (1984). Cell adhesion: Competition between nonspecific repulsion and specific binding. *Biophys. J.* 45, 1051–1064.
- BYRN, R. A., SEKIGAWA, I., CHAMOW, S. M., JOHNSON, J. S., GREGORY, T. J., CAPON, D. J., and GROOPMAN, J. E. (1989). Characterization of

*in vitro* inhibition of human immunodeficiency virus by purified recombinant CD4. *J. Virol.* **63**, 4370–4375.

- CLARK, S. J., SAAG, M. S., DECKER, W. D., CAMPBELL-HILL, S., ROBER-SON, J. L., VELDKAMP, P. J., KAPPES, J. C., HAHN, B. H., and SHAW, G. M. (1991). High titers of cytopathic virus in plasma of patients with symptomatic primary HIV-1 infection. *N. Engl. J. Med.* **324**, 954–960.
- COOMBS, R. W., COLLIER, A. C., ALLAIN, J.-P., NIKORA, B., LEUTHER, M., GJERSET, G. F., and COREY, L. (1989). Plasma viremia in human immunodeficiency virus infection. *N. Engl. J. Med.* **321**, 1626– 1631.
- DAAR, E. S., MOUDGIL, T., MEYER, R. D., and Ho, D. D. (1991). Transient high levels of viremia in patients with primary human immunodeficiency virus type 1 infection. N. Engl. J. Med. 324, 961–964.
- DEMBO, M., and BELL, G. I. (1987). The thermodynamics of cell adhesion. Curr. Top. Membr. Transp. 29, 71–89.
- DIMITROV, D. S., HILLMAN, K., MANISCHEWITZ, J., BLUMENTHAL, R., and GOLDING, H. (1992). Kinetics of sCD4 binding to cells expressing HIV-1 envelope glycoprotein. *J. Virol.* **66**, 132–138.
- EARL, P. L., DOMS, R. W., and Moss, B. (1990). Oligomeric structure of the human immunodeficiency virus type 1 envelope glycoprotein. *Proc. Natl. Acad. Sci. USA* **87**, 648–652.
- EFRON, B., and TIBSHIRANI, R. (1991). Statistical data analysis in the computer age. *Science* **253**, 390–395.
- FENOUILLET, E., CLERGET-RASLAIN, B., GLUCKMAN, J. C., GUÉTARD, D., MONTAGNIER, L., and BAHRAOUI, E. (1989). Role of N-linked glycans in the interaction between the envelope glycoprotein of human immunodeficiency virus and its cellular CD4 receptor. J. Exp. Med. 169, 807–822.
- FENYÖ, E. M., ALBERT, J., and ÅSJÖ, B. (1989). Replicative capacity, cytoplasmic effect and cell tropism of HIV. AIDS (Suppl. 1) 3, S5– S12.
- FURMAN, P. A., PAINTER, G., WILSON, J. E., CHENG, N., and HOPKINS, S. (1991). Substrate inhibition of the human immunodeficiency virus type 1 reverse transcriptase. *Proc. Natl. Acad. Sci. USA* 88, 6013– 6017.
- GELDERBLOM, H. R., REUPKE, H., and PAULI, G. (1985). Loss of envelope antigens of HTLV-III/LAV, a factor in AIDS pathogenesis? *Lancet* 2, 1016–1017.
- GELDERBLOM, H. R., HAUSMANN, E. H. S., ÖZEL, M., PAULI, G., and KOCH, M. A. (1987). Fine structure of human immunodeficiency virus (HIV) and the immunolocalization of structural proteins. *Virol*ogy **156**, 171–176.
- GELDERBLOM, H. R., ÖZEL, M., HAUSMAN, E. H. S., WINKEL, T., PAULI, G., and KOCH, K. A. (1988). Fine structure of human immunodeficiency virus (HIV), immunolocalization of structural proteins and virus-cell relation. *Micron Microsc.* **19**, 41–60.
- GELDERBLOM, H. R. (1991). Assembly and morphology of HIV: Potential effect of structure on function. *AIDS* **5**, 617–638.
- GERWIN, B. L., and LEVIN, J. G. (1977). Interactions of murine leukemia virus core components: Characterization of reverse transcriptase packaged in the absence of 70S genomic RNA. *J. Virol.* 24, 478–488.
- GOFF, S., TRAKTMAN, P., and BALTIMORE, D. (1981). Isolation and properties of Moloney Murine Leukemia Virus mutants: Use of a rapid assay for release of virion reverse transcriptase. *J. Virol.* **38**, 239– 248.
- GOFF, S. (1990). Retroviral reverse transcriptase: synthesis, structure, and function. J. Acquired Immune Defic. Syndr. 3, 817–831.
- GOODMAN, N. C., and SPIEGELMAN, S. (1971). Distinguishing reverse transcriptase of an RNA tumor virus from other known DNA polymerases. *Proc. Natl. Acad. Sci. USA* 68, 2203–2206.

- HART, T. K., KIRSH, R., ELLENS, H., SWEET, R. W., LAMBERT, D. M., PETTEWAY, S. R., LEARY, J., and BUGELSKI, P. J. (1991). Binding of soluble CD4 proteins to human immunodeficiency virus type 1 and infected cells induces release of envelope glycoprotein gp120. *Proc. Natl. Acad. Sci. USA* 88, 2189–2193.
- HELGSTRAND, E., ERIKSSON, B., JOHANSSON, N. G., LANNERÖ, B., LARSSON, A., MISIORNY, A., NORÉN, J. O., SJÖBERG, B., STENBERG, K., STENING, G., STRIDH, S., ÖBERG, B., ALENIUS, S., and PHILIPSON, L. (1978). Trisodium Phosphonoformate, a new antiviral compound. *Science* **201**, 819–821.
- Ho, D. D., MOUDGIL, T., and ALAM, M. (1989). Quantitation of human immunodeficiency virus type 1 in the blood of infected persons. *N. Engl. J. Med.* **321**, 1621–1625.
- HögLund, S., ÖFVERSTEDT, L.-G., NILSSON, Å., ÖZEL, M., WINKEL, TH., SKOGLÄND, U., and GELDERBLOM, H. (1991). Analysis of the assembly of the HIV core by electron microscope tomography. *In* "Retroviral Proteases, Control of Maturation and Morphogenesis" (L. H. Pearl, Ed.), pp. 149–157. Macmillan, London.
- KACIAN, D. L., WATSON, K. F., BURNY, A., and SPIEGELMAN, S. (1971). Purification of the DNA polymerase of avian myleoblastosis virus. *Biochem. Biophys. Acta* 246, 365–383.
- KRAKOWER, J. M., BARBACID, M., and AARONSON, S. A. (1977). Radioimmunoassay for mammalian type C viral reverse transcriptase. J. Virol. 22, 331–339.
- LAYNE, S. P., SPOUGE, J. L., and DEMBO, M. (1989). Quantifying the infectivity of human immunodeficiency virus. *Proc. Natl. Acad. Sci. USA* 86, 4644–4648.
- LAYNE, S. P., MERGES, M. L., DEMBO, M., SPOUGE, J. L., and NARA, P. L. (1990). HIV requires multiple gp120 molecules for CD4-mediated infection. *Nature* **346**, 277–279.
- LAYNE, S. P., MERGES, M. L., SPOUGE, J. L., DEMBO, M., and NARA, P. L. (1991). Blocking of human immunodeficiency virus infection depends on cell density and viral stock age. *J. Virol.* **65**, 3293– 3300.
- LAYNE, S. P., and DEMBO, M. (1992). The auto-regulation model: A unified concept of how HIV regulates its infectivity, pathogenesis and persistence. *Int. Rev. Immunol.* **8**, 1–32.
- LOONEY, D. J., HAYASHI, S., NICKLAS, M., REDFIELD, R. R., BRODER, S., WONG-STAAL, F., and MITSUYA, H. (1990). Differences in the interaction of HIV-1 and HIV-2 with CD4. *J. Acquired Immune Defic. Syndr.* **3**, 649–657.
- LORI, F., SCOVASSI, A. I., ZELLA, D., ACHILLI, G., CATTANEO, E., CASOLI, C., and BERTAZZONI, U. (1988). Enzymatically active forms of reverse transcriptase of the human immunodeficiency virus. *AIDS Res. Hum. Retroviruses* **4**, 393–398.
- LU, S., PUTNEY, S. D., and ROBINSON, H. L. (1992). Human immunodeficiency virus type 1 entry into T cells: More-rapid escape from an anti-V3 loop than from an antireceptor antibody. *J. Virol.* **66**, 2547– 2550.
- LUFTIG, R., IKUTA, K., BU, M., and CALKINS, P. (1990). Terminal stages of retrovirus morphogenesis. *In* "Retroviral Proteases: Maturation and Morphogenesis" (Pearl, L. H., Ed.), pp. 141–148. Macmillan, London.
- MAJUMDAR, C., ABBOTTS, J., BRODER, S., and WILSON, S. H. (1988). Studies on the mechanism of human immunodeficiency virus reverse transcriptase. J. Biol. Chem. 263, 15657–15665.
- MCDOUGAL, J. S., MARTIN, L. S., CORT, S. P., MOZEN, M., HELDEBRANT, C. M., and EVATT, B. L. (1985). Thermal inactivation of the acquired immunodeficiency syndrome virus, human T lymphotropic virus-III/lymphadenopathy-associated virus, with special reference to antihemophilic factor. J. Clin. Invest. **76**, 875–877.

MCKEATING, J. A., MCKNIGHT, A., and MOORE, J. P. (1991). Differential

loss of envelope glycoprotein gp120 from virions of human immunodeficiency virus type 1 isolates: Effects on infectivity and neutralization. J. Virol. **65**, 852–860.

- MEEK, T. D., DAYTON, B. D., METCALF, B. W., DREYER, G. B., STRICKLER, J. E., GORNIAK, J. G., ROSENBERG, M., MOORE, M. L., MAGAARD, V. W., and DEBOUCK, C. (1989). Human immunodeficiency virus 1 protease expressed in *Escherichia coli* behaves as a dimeric aspartic protease. *Proc. Natl. Acad. Sci. USA* 86, 1841– 1845.
- MIZRAHI, V., LAZAZUS, G. M., MILES, L. M., MEYERS, C. A., and DE-BOUCK, C. (1989). Recombinant HIV-1 reverse transcriptase: Purification, primary structure, and polymerase/ribonuclease H activities. *Arch. Biochem. Biophys.* 273, 347–358.
- MODROW, S., HAHN, B. H., SHAW, G. M., GALLO, R. C., WONG-STAAL, F., and WOLF, H. (1987). Computer-assisted analysis of envelope protein sequence of seven human immunodeficiency virus isolates: Prediction of antigenic epitopes in conserved and variable regions. J. Virol. 61, 570–578.
- MOORE, J. P., and JARRETT, R. F. (1988). Sensitive ELISA for the gp120 and gp160 surface glycoproteins of HIV-1. AIDS Res. Hum. Retroviruses 4, 369–379.
- MOORE, J. P., WALLACE, L. A., FOLLETT, E. A. C., and MCKEATING, J. A. (1989). An enzyme-linked immunosorbent assay for antibodies to the envelope glycoproteins of divergent strains of HIV-1. *AIDS* 3, 155–163.
- MOORE, J. P., MCKEATING, J. A., WEISS, R. A., and SATTENTAU, Q. J. (1990). Dissociation of gp120 from HIV-1 virions induced by soluble CD4. *Science* 250, 1139–1142.
- NARA, P. L., DUNLOP, N. M., ROBEY, W. G., CALLAHAN, R., and Fis-CHINGER, P. J. (1987a). Lipoprotein-associated oncornavirus-inactivating factor in the genus *Mus:* Effects on murine leukemia viruses of laboratory and exotic mice. *Cancer Res.* 47, 667–672.
- NARA, P. L., HATCH, W. C., DUNLOP, N. M., ROBEY, W. G., ARTHUR, L. O., GONDA, M. A., and FISCHINGER, P. J. (1987b). Simple, rapid, quantitative, syncytium-forming microassay for the detection of human immunodeficiency virus neutralizing antibody. *AIDS Res. Hum. Retroviruses* **3**, 283–302.
- NARA, P. L., and FISCHINGER, P. J. (1988). Quantitative infectivity assay for HIV-1 and -2. *Nature* 332, 469–470.
- ÖBERG, B. (1983). Antiviral effects of phosphonoformate (PFA, foscarnet sodium). *Pharmacol. Ther.* **19**, 387–415.
- ÖZEL, M., PAULI, G., and GELDERBLOM, H. R. (1988). The organization of the envelope projections on the surface of HIV. *Arch. Virol.* 100, 255–266.
- PANET, A., BALTIMORE, D., and HANAFUSA, T. (1975). Quantitation of avian RNA tumor virus reverse transcriptase by radioimmunoassay. J. Virol. 16, 146–152.
- PANET, A., and KRA-Oz, Z. (1978). A competition immunoassay for characterizing the reverse transcriptase of mammalian RNA tumor viruses. *Virology* 89, 95–101.
- PANTALEO, G., GRAZIOSI, C., BUTINI, L., PIZZO, P. A., SCHNITTMAN, S. M., KOTLER, D. P., and FAUCI, A. S. (1991). Lymphoid organs function as major reservoirs for human immunodeficiency virus. *Proc. Natl. Acad. Sci. USA* 88, 9838–9842.
- PLUNKETT, M. L., SANDERS, M. E., SELVARAJ, P., DUSTIN, M. L., and SPRINGER, T. A. (1987). Rosetting of activated human T lymphocytes with autologous erythrocytes. J. Exp. Med. 165, 664–676.
- SALMEEN, I., RAMAI, L., LUFTIG, R. B., LIEBES, L., RETZEL, E., RICH, M., and McCORMICK, J. J. (1976). Hydrodynamic diameters of murine mammary, rous sarcoma, and feline leukemia RNA tumor viruses: Studies by laser beat frequency light-scattering spectroscopy and electron microscopy. J. Virol. 17, 584–596.

- SANDSTROM, E. G., KAPLAN, J. C., BYINGTON, R. E., and HIRSCH, M. S. (1985). Inhibition of human T-cell lymphotropic virus type III *in vitro* by phosphonoformate. *Lancet* 1, 1480–1482.
- SCHAWALLER, M., SMITH, G. E., SKEHEL, J. J., and WILEY, D. C. (1989). Studies with crosslinking reagents on the oligomeric structure of the env glycoprotein of HIV. *Virology* **172**, 367–369.
- SHARP, D. G., and BEARD, J. W. (1950). Size and density of polystyrene particles measured by ultracentrifugation. J. Biol. Chem. 185, 247–253.
- SHAW, G. M., HAHN, B. H., ARYA, S. K., GROOPMAN, J. E., GALLO, R. C., and WONG-STAAL, F. (1984). Molecular characterization of human

T-cell leukemia (lymphotropic) virus type III in the acquired immune deficiency syndrome. *Science* **226**, 1165–1171.

- SPRINGER, T. A. (1990). Adhesion receptors of the immune system. *Nature* **346**, 425–434.
- VENABLE, J. H., and COGGESHALL, R. (1965). A simplified lead citrate stain for use in electron microscopy. J. Cell Biol. 26, 407–440.
- VERONESE, F. M., COPELAND, T. D., DEVICO, A. L., RAHMAN, R., OROSZ-LAN, S., GALLO, R. C., and SARNGADHARAN, M. G. (1986). Characterization of highly immunogenic p66/p51 as the reverse transcriptase of HTLV-III/LAV. *Science* 231, 1289–1291.
- VOGT, P. K. (1965). Avian tumor viruses. Adv. Virus Res. 2, 294-385.