Inhibition of Virus Production in Peripheral Blood Mononuclear Cells from Human Immunodeficiency Virus (HIV) Type 1-Seropositive Donors by Treatment with Recombinant HIV-Like Particles

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We have previously reported on the assembly of recombinant human immunodeficiency virus (HIV)-like particles that contain *gag* structural proteins and present *env* glycoproteins gp120 and gp41 on their surfaces (O. Haffar, J. Garriques, B. Travis, P. Moran, J. Zarling, and S.-L. Hu, *J. Virol.* 64:2653-2659, 1990). On the basis of their structures, we hypothesized that the recombinant particles would interfere with virus infection and tested our hypothesis *in vitro* by using peripheral blood mononuclear cells (PBMC) from HIV type 1-seropositive donors. Addition of the recombinant particles to PBMC concomitant with stimulation by anti-CD3 inhibited virus production, as determined by reduced levels of p24 in the culture supernatants. This inhibition of p24 production correlated with lower levels of cell-associated viral DNA. Several lines of evidence suggested that the recombinant particles exerted their antiviral effects primarily by inhibiting virus production from latently infected cells and not by inhibiting subsequent virus spread. Importantly, CD4⁺ T-cell stimulation by specific antigen or by anti-CD3 was not inhibited by treatment with the recombinant particles. This apparent selective inhibition of virus replication in infected PBMC represents a novel property of the recombinant HIV-like particles.

Recombinant human immunodeficiency virus (HIV)-like particles are generated when mammalian cells are infected with a vaccinia virus/HIV recombinant carrying both the *gag* and *env* genes of HIV (5, 6). The particles contain both *gag* and *env* structural proteins, are morphologically similar to the HIV virus, but are nonreplicating (5). When inoculated into rabbits, the recombinant particles elicited both HIV-specific humoral and cellular immunities (6). Since the recombinant particles presented *env* glycoproteins gp120 and gp41 on their surfaces, we hypothesized that they might also interfere with HIV replication.

To evaluate the putative antiviral properties of the recombinant HIV-like particles, we used peripheral blood mononuclear cells (PBMC) isolated from HIV type 1 (HIV-1)-seropositive donors. We selected this system because it provides heterologous endogenous virus isolates and is more biologically relevant than testing putative antiviral effects against one laboratory isolate of HIV-1 in a CD4⁺ cell line.

It has been shown that only a small fraction of CD4⁺ cells from healthy seropositive individuals (0.003 to 1%) harbor the provirus (23) in what appears to be a latent state (7). Studies on HIV-1 long terminal repeat (LTR)-directed gene expression suggested that virus could be induced in these PBMC cultures by either mitogenic (28) or nonmitogenic (4) stimulation of the infected CD4⁺ cells. Recent work (21a, 35) has shown that soluble anti-CD3 monoclonal antibody (MAb) is a very potent stimulatory signal for virus induction from latently infected cells. Soluble anti-CD3 MAb generates an acute but transient stimulatory signal (16) that is dependent on the presence of antigen-presenting cells (28) for induction of both T-cell proliferation and HIV-1 production.

In this report we present evidence to suggest that treatment with recombinant HIV-like particles inhibits HIV-1 virus production in anti-CD3-stimulated PBMC cultures from seropositive donors. This antiviral effect is not due to competition for the available CD4 molecules as was initially hypothesized but appears to be mediated by inhibiting induction of latent HIV-1.

MATERIALS AND METHODS

Cells and viruses. Monkey kidney cells BSC-40 were used for producing the recombinant particles, *gag* particles, and vaccinia virus. HIV-1 virus was inactivated by treatment with UV light and psoralen as described previously (33). UV-inactivated herpes simplex virus type 1 KOS strain was used in the activation of seronegative PBMC. All experiments were conducted with PBMC from either HIV-1-seropositive or HIV-1-seronegative donors as indicated.

MAbs. Anti-CD3 G19-4 (immunoglobulin G1) and anti-CD4 G17-2 (immunoglobulin G1) were described earlier (18). The G17-2 MAb is specific for the gp120-binding domain of CD4 and blocks the binding of purified gp120 to CD4⁺ cells (19). Anti-HIV-1 MAbs used were reactive with p24 (Genetic Systems Inc.) and gp120 (Genetic Systems Inc.) (19, 29).

Recombinant particles and *gag* particles. The recombinant particles were produced in BSC-40 cells infected with a vaccinia virus/HIV recombinant (5) carrying both the *gag* and *env* genes under the transcriptional control of separate 7.5-kDa vaccinia virus early promoters (10, 32a). For generating *gag* particles, BSC-40 cells were infected with a vaccinia virus/HIV recombinant (v-*gag*2) carrying the *gag* and protease genes (5, 11). To deplete the residual vaccinia virus from our particle preparations, the final particle fractions were incubated with rabbit anti-vaccinia virus polyclonal sera overnight at 4°C and then were incubated with

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goat anti-rabbit antibody-coated magnetic beads (Advanced Magnetics Inc.) for 1 h at 22°C. The beads were then sedimented by centrifugation at $500 \times g$. This procedure allowed the depletion of approximately 10^3 PFU of vaccinia virus per ml. The remaining vaccinia virus in the recombinant particle and *gag* particle preparations was inactivated by exposure to UV light. Inactivation was confirmed by plaque assay.

HIV infectivity experiments. PBMC from asymptomatic HIV-1-seropositive donors were isolated by Ficoll-Hypaque (Pharmacia) density gradient centrifugation. The PBMC were depleted of CD8⁺ T cells by incubation with anti-CD8 MAb (15a) and rabbit complement (Pel-Freez, Brown Deer, Wis.). The resulting cells were seeded into 24-well plates at 10^6 cells per well in 0.5 ml of culture medium (RPMI 1640 supplemented with 10% human serum). Duplicate wells received various concentrations of recombinant particles, inactivated HIV-1, *gag* particles, or inactivated vaccinia virus (see individual experiments). All wells were then supplemented with 0.5 ml of culture medium containing anti-CD3 (MAb G19-4) in solution at 1 μ g/ml final concentration as well as interleukin-2 (IL-2) (Pharmacia) at a final concentration of 5%. In the experiment presented in Fig. 5, cells were washed free of treatment 24 h after activation and then supplemented with culture medium containing anti-CD3 and IL-2 as described above. For the time course experiments (Fig. 6), recombinant particles were added at various times subsequent to the addition of the anti-CD3 as indicated.

Four days after activation, the culture media were withdrawn from the wells and the cells were washed with phosphate-buffered saline (PBS)-human serum and replaced with fresh culture media. A sample of cells from each well was collected and assayed for proliferation as described below. The remaining cells were cultured further for 3 days in the presence of 5% IL-2, at which time the growth medium was collected and assayed for p24 content by specific antigen capture (enzyme immunoassay) (Genetic Systems Inc.) (35). For evaluation of cell-associated HIV-1 DNA, cells from duplicate wells (3×10^6 cells per well) were harvested and pooled and the DNA fractions were prepared for analysis by polymerase chain reaction (PCR) (26) as described below.

Infection with cell-free virus and cocultivation experiments. CD8⁺-depleted PBMC from a seropositive donor were stimulated with anti-CD3 and cultured for 11 days in the presence of 5% IL-2 (source for donor cells and HIV-1-positive supernatants). Target cells were prepared by stimulating CD8⁺-depleted PBMC from seronegative donors with 3 μ g of phytohemagglutinin (PHA) per ml for 3 days. Target cells treated with recombinant particles (0, 5, or 50 μ g/ml) for 3 h at 37°C were washed repeatedly with 50% culture medium–50% PBS and incubated with HIV-1-positive supernatant (at 1:2) or cocultivated with HIV-1-positive donor cells at a ratio of 1/10 (donor cell-target cell). After 7 days, the culture medium was collected and p24 concentrations were determined by specific enzyme immunoassay.

Analysis of viral DNA content by PCR. Cell pellets (6×10^6) from pooled duplicate wells were resuspended in PCR lysis buffer (50 mM KCl, 10 mM Tris-HCl [pH 8.3], 2.5 mM MgCl₂ · 6H₂O, 0.1 mg of gelatin per ml, 0.45% Nonidet P-40, 0.45% Tween 20) containing 100 mg of proteinase K per ml and incubated for 1 h at 60°C with vigorous agitation every 20 min. The cells were then boiled for 15 min to denature the DNA and inactivate the proteinase K. DNA (equivalent to 1.5×10^5 cells) was amplified by using oligonucleotide primers specific for the U3 region of the HIV 3' LTR (5'

CAGATATCCACTGACCTTTGGATGGT 3' and 5' AGCA GCTGCTTATATGCAGC 3') or with oligonucleotide primers specific for the *vpr* gene (5' CAGAGGGAGCCACACAA TGAA 3' and 5' GATAAACAGCAGTTGTTGCAG 3'). To test relative starting amounts of DNA in the PCR reactions, samples were diluted fivefold (equivalent to 5×10^4 cells) and amplified by using oligonucleotide primers specific for the β -globin gene (5' GAAGAGCCAAGGACAGGTAC 3' and 5' CAACTTCATCCACGTTCCACC 3'). Amplification of each sample was conducted in duplicate for 30 cycles (95°C for 1 min and 60°C for 1 min), and then the samples were incubated at 60°C for 10 min. Amplified HIV DNA was visualized by oligomer hybridization (14) with the appropriate ³²P-end-labeled primers. The radiolabeled probes were prepared by T4 polynucleotide kinase treatment of the oligonucleotide primers (HIV_{LTR} 5' CAGATATCCACTGACCTTTGGATGGT 3' or HIV_{vpr} 5' CAGAGGGAGCCACA CAATGAA 3') in the presence of [γ -³²P]ATP. The radioactive probes were then applied to a G-50 Sephadex quick spin column (Pharmacia), and 2.5×10^5 cpm was added directly to 30 μ l of the amplified DNA in the presence of 60 mM NaCl–40 mM EDTA. Samples were incubated at 95°C for 5 min and then for 15 min at 55°C. The hybridized DNA was fractionated in a 10% acrylamide gel and visualized by autoradiography. The β -globin DNA was visualized by ethidium bromide staining.

Cell surface binding assay. PBMC from seronegative donors were isolated, depleted of CD8⁺ T cells as outlined above, and treated with various concentrations of recombinant particles or inactivated HIV for 2 h at 37°C (2×10^6 cells per treatment). The cells were washed with PBS–1% fetal bovine serum and incubated with a mixture of anti-HIV MAbs or fluorescein isothiocyanate (FITC)-conjugated anti-CD4 MAb (G17-2) at a final dilution of 1:100 for 30 min at 4°C. Cells incubated with the HIV-specific MAbs were washed with PBS and incubated with FITC-conjugated goat anti-mouse F(ab')₂ (Tago) at 1:100 for 30 min at 4°C. All cells were washed with PBS and fixed in 1% paraformaldehyde, and binding was analyzed by fluorescence-activated cell sorter.

Calcium mobilization and measurement of [Ca²⁺]_i. PBMC from a seronegative donor were incubated with 10 and 50 μ g of recombinant particles or inactivated HIV per ml for 2 h at 37°C. During the final 45 min of the incubation, the cells were loaded with indo-1 (24) in the presence of phycoerythrin-conjugated anti-CD8, anti-CD20, anti-CD11b, and anti-major histocompatibility complex class II MAbs as described previously (18). The addition of the MAbs allowed the gating on the unstained CD4⁺ cells. Cells were then washed with PBS and stimulated with 10 μ g of anti-CD3 (G19-4) per ml. Increase in intracellular calcium concentration ([Ca²⁺]_i) in CD4⁺ T cells was measured immediately after stimulation by determining the mean indo-1 violet/blue fluorescence ratio as a function of time, using flow cytometry (24). For measuring maximal inhibition of calcium mobilization, CD4 molecules were aggregated at the cell surface by cross-linking them with anti-CD4 MAb (25) before stimulation with G19-4.

Lymphoproliferative assay. Four days after stimulation with anti-CD3 as outlined above, PBMC from seropositive donors were seeded into 96-well plates at 10^5 cells per well. PBMC from seronegative donors stimulated with 2×10^5 PFU of UV-inactivated HSV-1 per ml for 7 days were also seeded into 96-well plates. All cells were then incubated with tritiated thymidine ([³H]TdR) at 1.0 mCi per well for 6 h and harvested, and the incorporated radioactivity was measured by liquid scintillation counting.

FIG. 1. Inhibition of virus production by treatment with recombinant particles. (A) PBMC from three seropositive donors (Z29, Z30, and Z39) were stimulated with anti-CD3 and treated with increasing concentrations of recombinant particles (0, 0.1, 1, and 10 $\mu\text{g/ml}$). The effect of treatment with inactivated HIV was also evaluated. Inhibition of HIV production is presented as the percent decrease in extracellular p24 (y axis) in treated versus untreated cultures. The concentrations of p24 in culture supernatants after stimulation in the absence of treatment were 16.6 ng/ml (Z29), 6.6 ng/ml (Z30), and 1.9 ng/ml (Z39). These values correspond to 100% induction. (B) The effect of UV-inactivated vaccinia virus on HIV replication in PBMC from donor Z39 is presented in parallel with that of recombinant particles. The top x axis refers to the concentrations of recombinant particles ($\mu\text{g/ml}$) with respect to p24 content. The lower x axis refers to the concentrations of vaccinia virus (PFU/ml) in both preparations as indicated. The y axis is as described for panel A. The concentration of p24 in supernatant of the stimulated untreated culture was 8.7 ng/ml.

RESULTS

Inhibition of virus production. PBMC from three seropositive donors were depleted of CD8⁺ T cells and stimulated with soluble anti-CD3 MAb in the presence of 5% IL-2. As shown in Fig. 1, addition of the recombinant particles concomitant with the anti-CD3 inhibited virus production in PBMC from all three donors, as determined by reduced levels of p24 in the culture media. Similar inhibition was achieved by using inactivated HIV.

Since the recombinant particles were generated by infection of cells with vaccinia virus/HIV recombinant, the isolated particle fractions contained residual vaccinia virus. Therefore, we evaluated the contribution of vaccinia virus to the overall antiviral effect of the recombinant particles. Vaccinia virus was harvested from the culture supernatants of BSC-40 cells infected with the parental vaccinia virus (v-NY). PBMC from donor Z39 were stimulated with anti-CD3 and treated with various concentrations of recombinant particles or UV-inactivated vaccinia virus. At concentrations equivalent to those in the recombinant particle preparation (lower x axis), inactivated vaccinia virus effected low inhibition of HIV production relative to the recombinant particles (e.g., 30 versus 90%, respectively, at their highest concentrations) (Fig. 1B). Similar results were obtained by using cells from other donors (see Fig. 3 and 5; also data not shown). The difference in inhibition exerted by the recombinant particles on PBMC from donor Z39 presented in Fig. 1A versus 1B was not surprising since variability in response to anti-CD3 (compare the p24 values in the legend to Fig. 1A with those shown in Fig. 1B) and to treatments with recombinant particles and inactivated HIV was routinely observed with PBMC derived from different blood samples.

The measured reduction in p24 production could have resulted from either a lower number of infected cells or from lower expression levels in infected cells. Therefore, we

evaluated the level of infection in the cultures by assaying for cell-associated viral DNA using quantitative PCR. Primers specific to the 3' LTR and the *vpr* open reading frame of HIV were selected because they allowed the detection of complete as well as incomplete transcripts (34). As shown in the top and middle panels of Fig. 2, cultures treated with recombinant particles (samples 4 and 5) contained a lower amount of cell-associated HIV DNA than untreated cultures (samples 2 and 3) or vaccinia virus-treated cultures (samples 6 and 7). In parallel reactions, samples were amplified by using oligonucleotide primers specific for the β -globin gene to ensure that equivalent starting amounts of DNA were used (bottom panel). Analysis of unstimulated cultures (sample 1) established the basal level of cell-associated HIV DNA. Positive and negative controls for the PCR reactions were conducted by using known amounts of DNA from the infectious clone PNL4-3 (1) or DNA from uninfected PBMC, respectively (data not shown). Results from PCR experiments showed that the inhibition of p24 production correlated with reduction in the overall levels of cell-associated viral DNA. This finding suggests that inhibition of virus production after treatment with recombinant particles is primarily due to the reduction in total number of infected cells.

Binding to cell surface-associated CD4 is necessary for the antiviral effect of the recombinant particles. Previous reports suggested that the envelope glycoproteins of HIV-1 modulate the activation of T cells by binding to the CD4 receptor (17, 21). Therefore, we tested whether the *env* glycoproteins gp120 and gp41 presented on the surface of the recombinant particles are necessary for the antiviral effect. To address this question, particles devoid of *env* glycoproteins and composed only of *gag* proteins were generated from BSC-40 cells infected with vaccinia virus/HIV *gag* recombinant (5, 11). PBMC from donor Z29 were treated with various

FIG. 2. Analysis of cell-associated HIV DNA. PBMC from donor Z29 were stimulated with anti-CD3 in the absence (samples 2 and 3) or presence of 5 μ g of recombinant particles (samples 4 and 5) per ml or 10^5 PFU of inactivated vaccinia virus (samples 6 and 7) per ml. Each sample represents cells pooled from duplicate wells in the infectivity experiment, and, as shown, the PCR reactions were conducted in duplicate for each sample. Amplification of DNA was conducted by using oligonucleotide primers specific for HIV (LTR and *vpr*) or specific for β -globin. The HIV DNA fragments were oligohybridized, fractionated in 10% acrylamide gels, and visualized by autoradiography. The β -globin DNA was also fractionated in 10% acrylamide gels but was visualized by staining with ethidium bromide. Basal levels of HIV DNA from unstimulated PBMC are presented in sample 1.

concentrations of either recombinant particles or *gag* particles, normalized for their p24 content. As shown in Fig. 3, at 1 μ g/ml the *gag* particles exerted marginal inhibition of virus production compared with the particles containing the *env* glycoproteins (20 to 90%, respectively). For comparison, the inhibition achieved with 10^5 PFU of UV-inactivated vaccinia

virus per ml, equivalent to the vaccinia virus content in the highest concentration of *gag* particles used, is also presented. The *gag* particle preparation contained approximately 10-fold more residual vaccinia virus than the recombinant particle preparation.

The effect of *gag* particles on cell-associated HIV DNA levels was also evaluated. PBMC from donor Z29 were incubated with 2.5 μ g of recombinant particles or *gag* particles per ml or 10^5 PFU of UV-inactivated vaccinia virus per ml, and the DNA fractions were analyzed for HIV DNA by PCR. The HIV-specific primers used in this experiment were as described earlier. Figure 3 shows that, consistent with the p24 data, the *gag* particles had a marginal effect on the HIV DNA content compared with recombinant particle-treated samples (compare sample 4 with sample 3). Moreover, the effect of *gag* particles could be accounted for by the vaccinia virus in the preparation (compare sample 4 with sample 5). Analysis of β -globin DNA is presented in the lower panel and shows that equivalent amounts of DNA were used in the PCR reactions. Therefore, the presence of the *env* glycoproteins and presumably binding to CD4 were necessary for the antiviral effect of the recombinant particles.

Treatment with recombinant particles does not inhibit cell-cell or cell-free transmission of the virus. Reduction in the number of infected cells after treatment with the recombinant particles could have resulted from inhibition of virus spread, inhibition of virus induction, or both. Inhibition of virus spread could in turn be due to either blocking of the virus binding to CD4 or rendering target cells nonpermissive for infection. Therefore, to determine whether treatment with the recombinant particles was inhibiting virus spread in the cultures, we first tested the saturation of cell surface-associated CD4 molecules in treated cells. PBMC from a seronegative donor were incubated with various concentrations of recombinant particles or inactivated HIV-1 and then reacted with either anti-HIV MAb or anti-CD4 MAb. As

FIG. 3. Presence of the HIV-1 envelope glycoprotein is necessary for the antiviral effect of the recombinant particles. The effect of treatment of PBMC from donor Z29 with *gag* particles and recombinant particles on HIV-1 replication was evaluated by assaying the levels of released p24 (line graph) and the levels of cell-associated HIV-1 DNA (autoradiogram). The concentrations of *gag* particles and recombinant particles were normalized for their p24 contents. The effect of 10^5 PFU of inactivated vaccinia virus per ml is also presented. The concentration of p24 in supernatant of the stimulated untreated culture was 3.2 ng/ml. Amplification and visualization of DNA samples was as described in the legend to Fig. 2.

FIG. 4. Binding of the recombinant particles to CD4⁺ cells does not saturate the cell surface-associated CD4 molecules. Recombinant particles (A) or inactivated HIV-1 (B) were incubated with PBMC from a seronegative donor. The cell-associated HIV-1 proteins were visualized by incubation with a mixture of HIV-specific MAbs and FITC-conjugated goat anti-mouse antibody. Panels D, E, and F present the binding of FITC-conjugated anti-CD4 MAb (G17-2) to CD4⁺ cells after binding of 10 µg of recombinant particles per ml (D) or 10 or 100 µg of inactivated HIV per ml (E and F, respectively). Panel C presents the binding of anti-CD4 MAb to untreated cells.

shown in Fig. 4, binding of recombinant particles and inactivated virus to cells was dose dependent (panel A). Interestingly, the inactivated virus appeared to bind to cells more than did the recombinant particles (panel B), which was surprising since they contained equivalent amounts of gp120 (6). Importantly, preincubating cells with 10 µg of either recombinant particles or inactivated virus per ml did not alter the level of anti-CD4 binding to cells (panels D and E, respectively). This result showed that at the highest concentration used in our virus inhibition experiments (10 µg/ml; Fig. 1) the recombinant particles did not saturate the cell surface-associated CD4 molecules. Displacement of the particles or virus by anti-CD4 could not account for this result, since at higher concentrations of inactivated virus (100 µg/ml) significant inhibition of anti-CD4 binding was observed (Fig. 4F).

On the basis of the results with the CD4-binding study, we concluded that at the concentrations mostly used in our studies (1 and 5 µg/ml) the recombinant particles would not inhibit virus binding and entry into cells. To address this question directly, PBMC from two seronegative donors (D819 and D830) were stimulated with PHA and treated with various concentrations of recombinant particles before incubation with supernatants derived from an HIV-1-infected PBMC culture (Z29). Although the efficiency of the infection varied for the two target cells, as shown in Table 1, treatment with particles did not affect the ability of the cell-free virus to infect the target cells. The inhibition observed with treatment of D819 cells with 50 µg of particles per ml may be due partially to experimental variability (observed as well in the vaccinia virus-treated target cells [data not shown]) as well as to true inhibition, since at 50 µg/ml there is significant binding of particles to cell surface CD4 (Fig. 4).

To explore whether the recombinant particles were rendering CD4⁺ target cells nonpermissive for infection, cocultivation assays were performed. PHA-stimulated PBMC

TABLE 1. Lack of effect of treatment with recombinant particles on infection of CD4⁺ target cells with HIV-1

Treatment	Target cells (PHA blasts) ^a	Virus production (ng of p24/ml) ^b after treatment with recombinant particles (µg of p24/ml):		
		0	5	50
Infection with cell-free virus ^c	D819	173.25	180.95	101.55
	D830	2.42	1.92	5.83
Cocultivation with HIV-1-infected donor PBMC	D819 ^d	126	170.3	166.5
	D830	56	71.05	76.2

^a Target cells were prepared by activating PBMC from seronegative donors with PHA for 3 days and then incubating them with recombinant particles for 3 h at 37°C.

^b p24 concentrations were measured by using a specific enzyme immunoassay.

^c PBMC from a seropositive donor were activated with anti-CD3 and cultured for 11 days in the presence of 5% IL-2. The culture supernatant was harvested as the source of cell-free virus and added to growth media of target cells (1:2). The infected cells were used as donor cells in the cocultivation assay. Donor cell/target cell ratio was 1:10.

^d The amount of p24 produced by donor cells incubated in the absence of target cells was 0.58 ng/ml.

from the same seronegative donors (see above) were incubated with various concentrations of recombinant particles. The cells were then washed and cocultivated with activated virus-producing PBMC from a seropositive donor (Z29). Table 1 shows that cocultivation of virus-producing cells (donor cells) with untreated target cells allowed the virus infection to spread as determined by the observed increase in the levels of extracellular p24 from 0.58 ng/ml in the absence of target cells to 127 ng/ml with D819 cells and 56 ng/ml with D830 cells. Importantly, pretreating the target cells with either 5 or 50 µg of recombinant particles per ml did not decrease the efficiency of virus transmission, as determined by the extracellular levels of p24 compared with those of samples from untreated target cells. Taken with the results from the CD4-binding study, the cell-free virus infection data and the cocultivation data strongly suggest that the antiviral effect of the recombinant particles is not due to inhibition of virus spread in the cultures. This conclusion is further supported by our finding that treatment of PBMC from donor Z29 with particles during the initial 24 h of stimulation was sufficient to give approximately 84% inhibition of virus production (at the highest concentration of particles) (Fig. 5).

Recombinant particles affect early events in the virus induction process. The data presented in the previous section suggest that the recombinant particles affect the early stages of virus production from latently infected cells. To further characterize this response, we studied the temporal requirements for addition of recombinant particles relative to the stimulation signal. PBMC from a seropositive donor (Z31) were stimulated with anti-CD3, and then 1 µg of recombinant particles per ml was added at various times after the stimulation signal. Figure 6 shows that there was effective inhibition of virus production (approximately 85%) when the particles were added within 4 h, but not after 40 h, of the activation signal. Therefore, the early steps in the virus induction pathway appear to be most sensitive to the effect of the recombinant particles.

Recombinant particles do not inhibit T-cell activation. Virus induction and propagation in CD4⁺ cells is dependent on successful activation of the latently infected cells as well as target cells (27, 34). Since the antiviral effect of the recom-

FIG. 5. Inhibition of virus production can be affected by short-term incubation of PBMC with recombinant particles. PBMC from donor Z29 were incubated with recombinant particles (0, 0.1, 1, and 10 $\mu\text{g/ml}$) during the initial 24-h activation period. The cells were then washed extensively and supplemented with culture medium containing anti-CD3 and IL-2. Also presented are results from treatment with 10^4 PFU of UV-inactivated vaccinia virus per ml that correspond to those from residual vaccinia virus present in 10 μg of recombinant particles per ml. The p24 concentration in supernatant of the stimulated untreated culture was 11.8 ng/ml.

binant particles requires binding to the CD4 molecules, we investigated whether this interaction interfered with the stimulation of cells by anti-CD3 (25). An early event in the stimulation of T cells via the CD3-T-cell receptor (TCR) pathway is the increase in $[\text{Ca}^{2+}]_i$ by mobilization of intracellular stores as well as the increase in calcium influx into the cell (for a review, see reference 2). Therefore, the effect of treatment with recombinant particles on calcium mobilization was evaluated. As shown in Fig. 7A, binding of 10 μg of recombinant particles per ml did not alter the anti-CD3 induced increase in $[\text{Ca}^{2+}]_i$, whereas incubation of cells with 10 μg of inactivated HIV per ml showed a slight decrease in the level of calcium mobilization (panel B). The difference in effect between the recombinant particles and inactivated HIV could be related to the higher binding to CD4 detected

FIG. 6. Early addition of the recombinant particles is necessary for the antiviral effect. Recombinant particles (1 $\mu\text{g/ml}$) were added to PBMC from seropositive donor Z31 either concomitant with anti-CD3 (0 h) or at various times after the addition of the MAb (1, 2, 4, and 40 h). The inhibition of p24 production is presented. The p24 concentration in supernatant from the stimulated untreated culture was 2.9 ng/ml.

FIG. 7. Recombinant particles do not inhibit calcium mobilization in stimulated CD4^+ T cells. PBMC from a seronegative donor were treated with various concentrations of recombinant particles (A) or inactivated HIV (B) and loaded with the dye indo-1. Calcium mobilization after stimulation of CD4^+ T cells with anti-CD3 is presented as indo-1 ratio of violet/blue as a function of time. Maximum inhibition in $[\text{Ca}^{2+}]_i$ mobilization was evaluated by cross-linking the CD4 molecules with anti-CD4 MAb after treatment with 10 μg of recombinant particles per ml (C).

with inactivated virus (Fig. 4). This conclusion is supported by the result showing that 50 μg of recombinant particles per ml and 10 μg of inactivated HIV per ml had similar inhibition of calcium mobilization (compare panel A with panel B) as well as similar levels of binding to cells (Fig. 4A and B). Additionally, in Fig. 7C, incubation with 10 μg of recombinant particles per ml did not alter the subsequent maximal inhibition of $[\text{Ca}^{2+}]_i$ mobilization exerted by cross-linking of CD4 with anti-CD4 MAb (G17-2).

These experiments suggested that binding of the recombinant particles to the CD4^+ T cells did not alter their stimulation via the CD3-TCR complex. However, since these experiments addressed very early events in the activation pathway, we also evaluated the effect of recombinant particle binding on cell proliferation measured 4 days after stimulation. These studies were conducted with PBMC from two seropositive donors (Z29 and Z30) stimulated with anti-CD3 as well as with PBMC from two seronegative donors (D372 and D768) stimulated with specific antigen (inactivated herpes simplex virus type 1). In both cases, activation of T cells was mediated via the CD3-TCR complex. As shown in Fig. 8, treatment with various concentrations of recombinant particles that were effective in inhibit-

FIG. 8. Recombinant particles do not significantly alter the lymphoproliferative response of CD4^+ cells. PBMC from seropositive donors (Z29 and Z30) and seronegative donors (D372 and D768) were stimulated with anti-CD3 or inactivated herpes simplex virus type 1, respectively, in the presence of increasing concentrations of recombinant particles. Lymphoproliferative responses are presented as counts per minute of incorporated ^3H TdR.

ing HIV production did not significantly alter the proliferation of cells in response to anti-CD3 or a specific antigen stimulation signal (compare with Fig. 1). Taken together, these data suggest that inhibition in virus production did not result from alteration in the efficiency of overall CD4^+ T-cell activation.

DISCUSSION

In this report, we have shown that treatment of PBMC from HIV-1-infected seropositive donors with recombinant HIV-like particles inhibited the replication of virus in the cultures. Inhibition was also detected after treatment with UV-psoralen-inactivated HIV-1 but not with *gag* particles, suggesting that CD4 binding may be necessary for the antiviral effect. Inhibition of virus production correlated with the overall reduction in cell-associated HIV-1 DNA presumably resulting from a decreased number of infected cells. Since the antiviral effect was measured in cells from four seropositive donors, the response appears to be independent of the endogenous HIV-1 virus isolate. We have shown that a small percentage of virus inhibition in the recombinant particle-treated cultures resulted from the presence of residual amounts of vaccinia virus. This might account for the slightly higher efficacy of the recombinant particles relative to inactivated HIV-1 (Fig. 1). At concentrations of 10^4 to 10^5 PFU/ml, UV-inactivated vaccinia virus inhibited HIV replication by approximately 15 to 40% (Fig. 1B, 3A, and 5).

The lack of CD4 receptor saturation and the failure to inhibit infection of treated target cells with either cell-free or cell-associated virus strongly suggest that the recombinant particles (1 to 10 $\mu\text{g/ml}$) exert their inhibition at the level of virus induction and not virus spread. This conclusion is supported by results showing that inhibition of virus production was achieved by incubating infected PBMC with particles for only 24 h (Fig. 5). In that duration, only the first round of virus replication in the stimulated cells occurs.

T-cell activation has been shown to induce HIV-1 LTR-directed gene expression (4, 32) and is necessary for HIV-1 replication (27, 34). Molecular events responsible for the

activation of the HIV-1 LTR have been shown to involve binding of various nuclear factors to specific sequences dispersed throughout the LTR (12, 15, 20, 32). The most compelling evidence for association of nuclear factor binding with HIV-1 LTR activation relates to the NF- κB protein complex (20, 32). Translocation of this complex into the nucleus and binding to DNA is dependent on phosphorylation of the inhibitory protein I κB by protein kinase C (PKC) (3). However, recent evidence suggests that functional association of the translocated NF- κB complex with the HIV-1 LTR in T-cell clones involves specific modifications of the complex or the binding of other factors (8). Whether PKC is involved in the activity of other nuclear-binding proteins is unknown; however, a central role for PKC in HIV-1 LTR induction has been suggested (4). In our studies we induced HIV-1 production from latently infected PBMC by activating cells with soluble anti-CD3 MAb in the presence of exogenous IL-2. We have found this form of activation to be a potent inducer of HIV-1 (35) and clearly superior to activation with other mitogens (21a). Moreover, T-cell activation via the CD3 -TCR complex after stimulation with soluble anti-CD3 is thought to closely resemble antigen stimulation of cells as it occurs in vivo.

Stimulation of CD4^+ T cells by engagement of the TCR- CD3 - CD4 complex is in part mediated by the CD4 -associated tyrosine kinase pp56^{Lck} (17) and results in the breakdown of phosphatidylinositol-phosphate (PIP_2) to generate diacylglycerol (DAG) and inositol phosphate-3 (IP_3). DAG in turn activates PKC, whereas IP_3 is involved in mobilization of intracellular calcium. Recent evidence shows that the breakdown of PIP_2 is due to the enzymatic activity of phosphatidylinositol-phospholipase C ($\text{PLC}\gamma_1$), which is regulated via tyrosine phosphorylation during pp56^{Lck} activation (13). Cross-linking of the CD4 molecules at the cell surface with anti- CD4 MAb and a second antibody abrogated the activation of T cells with anti- CD3 (25). This inhibitory effect of anti- CD4 on T-cell activation was duplicated by cross-linking CD4 with HIV gp120 and anti-gp120 MAb (17) or HIV patient immunoglobulin G fractions (21) and resulted from uncoupling of the CD4 and pp56^{Lck} (17). On the basis of the multivalent structure of the recombinant particles with respect to gp120 presentation, we hypothesized that binding to CD4^+ cells would result in cross-linking of the cell surface-associated CD4 and inhibition of T-cell activation. Our results from the calcium mobilization experiments supported this hypothesis with higher concentrations of particles or inactivated virus (Fig. 6). However, at lower concentrations, incubation with recombinant particles did not inhibit the anti- CD3 -induced $[\text{Ca}^{2+}]_i$ mobilization, nor did it significantly inhibit the proliferation of PBMC in response to either anti- CD3 or specific antigen. The lack of inhibition of proliferation by the recombinant particles in the anti- CD3 -stimulated cultures may be due in part to the addition of 5% IL-2 to the growth medium (22). This supplementation was necessary in order to account for differential sensitivity of PBMC from various donors to soluble anti- CD3 stimulation. Our results are in agreement with those of Hofmann et al. (9) who showed that incubation of PBMC from seronegative donors with low concentrations of inactivated HIV did not affect anti- CD3 -mediated increase in $[\text{Ca}^{2+}]_i$. However, Hofmann et al. (9) also showed that binding of inactivated HIV virus to the PBMC did inhibit the inositol phospholipid metabolism and subsequent PKC activation without affecting $[\text{Ca}^{2+}]_i$ mobilization. Recent evidence has shown that pp56^{Lck} is involved in the activation of other second-messenger systems in addition to $\text{PLC}\gamma_1$, such

as serine-threonine kinase Raf-1 (31) and phosphatidylinositol-3 kinase (30). Therefore, it is possible that binding of the recombinant particles to cell surface-associated CD4 may affect the induction of HIV-1 by modulating the activation by pp56^{lck} of one or a combination of second messengers. Clearly, delineating the complicated cascade of reactions resulting from activation of T cells via the TCR-CD3-CD4-pp56^{lck} complex would be important to our understanding of cell surface-mediated events necessary for induction of latent HIV.

We have shown that binding of recombinant HIV-like particles to CD4⁺ cells in PBMC cultures from seropositive donors inhibited the induction of virus from latently infected cells. This novel antiviral property, together with the immunogenic properties (6) of the recombinant HIV-like particles, may define a new application in the therapeutic regulation of AIDS.

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REFERENCES

- Adachi, A., H. E. Gendelman, S. Koenig, T. Folks, R. Willey, A. Rabson, and M. A. Martin. 1986. Production of acquired immunodeficiency syndrome-associated retrovirus in human and non-human cells transfected with an infectious molecular clone. *J. Virol.* **59**:284–291.
- Gardner, P. 1989. Calcium and T lymphocyte activation. *Cell* **59**:15–20.
- Ghosh, S., and D. Baltimore. 1990. Activation *in vitro* of NF- κ B by phosphorylation of its inhibitor I κ B. *Nature (London)* **344**:678–682.
- Gruters, R. A., S. A. Otto, B. J. M. Al, A. J. Verhoeven, C. L. Verweij, R. A. W. Van Lier, and F. Miedema. 1991. Non-mitogenic T cell activation signals are sufficient for induction of human immunodeficiency virus transcription. *Eur. J. Immunol.* **21**:167–172.
- Haffar, O., J. Garrigues, B. Travis, P. Moran, J. Zarling, and S.-L. Hu. 1990. Human immunodeficiency virus-like, nonreplicating gag-env particles assemble in a recombinant vaccinia virus expression system. *J. Virol.* **64**:2653–2659.
- Haffar, O. K., M. D. Smithgall, P. A. Moran, B. M. Travis, J. M. Zarling, and S.-L. Hu. 1991. HIV-specific humoral and cellular immunity in rabbits vaccinated with recombinant human immunodeficiency virus-like gag-env particles. *Virology* **183**:487–495.
- Harper, M. E., L. M. Marselle, R. C. Gallo, and F. Wong-Staal. 1986. Detection of lymphocytes expressing human T-lymphotropic virus type III in lymph nodes and peripheral blood from infected individuals by *in situ* hybridization. *Proc. Natl. Acad. Sci. USA* **83**:772–776.
- Hazan, U., D. Thomas, J. Alcamí, F. Bachelier, N. Israel, H. Yssel, J. L. Virelizier, and F. Arenzana-Seisdedos. 1990. Stimulation of a human T-cell clone with anti-CD3 or tumor necrosis factor induces NF- κ B translocation but not human immunodeficiency virus 1 enhancer-dependent transcription. *Proc. Natl. Acad. Sci. USA* **87**:7861–7865.
- Hofmann, B., P. Nishanian, R. L. Baldwin, P. Insixiangmay, A. Nel, and J. L. Fahey. 1990. HIV inhibits the early steps of lymphocyte activation, including initiation of inositol phospholipid metabolism. *J. Immunol.* **145**:3699–3705.
- Hu, S.-L., S. G. Kosowski, and J. M. Dalrymple. 1986. Expression of AIDS virus envelope gene in recombinant vaccinia viruses. *Nature (London)* **320**:537–540.
- Hu, S.-L., B. M. Travis, J. Garrigues, J. M. Zarling, P. Sridhar, T. Dykers, J. W. Eichberg, and C. Alpers. 1990. Processing, assembly, and immunogenicity of human immunodeficiency virus core antigens expressed by recombinant vaccinia virus. *Virology* **179**:321–329.
- Jones, K. A., J. T. Kadonaga, P. A. Luciw, and R. Tjian. 1986. Activation of the AIDS retrovirus promoter by the cellular transcription factor, Sp1. *Science* **232**:755–759.
- Kanner, S. B., T. J. Kavanagh, A. Grossmann, S.-L. Hu, J. B. Bolen, P. S. Rabinovitch, and J. A. Ledbetter. 1992. Sulfhydryl oxidation down-regulates T-cell signaling and inhibits tyrosine phosphorylation of phospholipase C γ 1. *Proc. Natl. Acad. Sci. USA* **89**:300–304.
- Kellogg, D. E., and S. Kwok. 1990. Detection of human immunodeficiency virus. In M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White (ed.), *PCR protocols: a guide to methods and applications*, p. 337–347. Academic Press, Inc., San Diego, Calif.
- Kliwer, S., J. Garcia, L. Pearson, E. Soultanakis, A. Dasgupta, and R. Gaynor. 1989. Multiple transcriptional regulatory domains in the human immunodeficiency virus type 1 long terminal repeat are involved in basal and E1A/E1B-induced promoter activity. *J. Virol.* **63**:4616–4625.
- Ledbetter, J., et al. Unpublished data.
- Ledbetter, J. A., L. E. Gentry, C. H. June, P. S. Rabinovitch, and A. F. Purchio. 1987. Stimulation of T cells through the CD3/T-cell receptor complex: role of cytoplasmic calcium, protein kinase C translocation, and phosphorylation of pp60^{c-src} in the activation pathway. *Mol. Cell. Biol.* **7**:650–656.
- Ledbetter, J. A., C. H. June, S.-L. Hu, and O. W. Press. 1990. Regulation of cytoplasmic calcium concentration in T cells. In C. A. Janeway, J. Sprent, and E. Sercarz (ed.), *Immunogenicity*, p. 113–123. Alan R. Liss Inc., New York.
- Ledbetter, J. A., C. H. June, P. S. Rabinovitch, A. Grossmann, T. T. Tsu, and J. B. Imboden. 1988. Signal transduction through CD4 receptors: stimulatory vs. inhibitory activity is regulated by CD4 proximity to the CD3/T cell receptor. *Eur. J. Immunol.* **18**:525–532.
- Linsley, P. S., J. A. Ledbetter, E. Kinney-Thomas, and S.-L. Hu. 1988. Effects of anti-gp120 monoclonal antibodies on CD4 receptor binding by the env protein of human immunodeficiency virus type 1. *J. Virol.* **62**:3695–3702.
- Lu, Y. C., N. Touzjian, M. Stenzel, T. Dorfman, J. G. Sodroski, and W. A. Haseltine. 1991. The NF κ B independent cis-acting sequences in HIV-1 LTR responsive to T-cell activation. *J. Acquired Immune Defic. Syndr.* **4**:173–177.
- Mittler, R. S., and M. K. Hoffmann. 1989. Synergism between HIV gp120 and gp120-specific antibody in blocking human T cell activation. *Science* **245**:1380–1382.
- Moran, P., et al. Unpublished data.
- Oyaizu, N., N. Chirmule, V. S. Kalyanaraman, W. W. Hall, R. Pahwa, M. Shuster, and S. Pahwa. 1990. Human immunodeficiency virus type 1 envelope glycoprotein gp120 produces immune defects in CD4⁺ T lymphocytes by inhibiting interleukin 2 mRNA. *Proc. Natl. Acad. Sci. USA* **87**:2379–2383.
- Psallidopoulos, M. C., S. M. Schnittman, L. M. Thompson III, M. Baseler, A. S. Fauci, H. C. Lane, and N. P. Salzman. 1989. Integrated proviral human immunodeficiency virus type 1 is present in CD4⁺ peripheral blood lymphocytes in healthy seropositive individuals. *J. Virol.* **63**:4626–4631.
- Rabinovitch, P. S., C. H. June, A. Grossmann, and J. A. Ledbetter. 1986. Heterogeneity among T cells in intracellular free calcium responses after mitogen stimulation with PHA or anti-CD3. Simultaneous use of indo-1 and immunofluorescence with flow cytometry. *J. Immunol.* **137**:952–961.
- Rosoff, P. M., S. J. Burakoff, and J. L. Greenstein. 1987. The role of the L3T4 molecule in mitogen and antigen-activated signal transduction. *Cell* **49**:845–853.
- Saiki, R. K., S. Scharf, F. Faloona, K. B. Mullis, G. T. Horn, H. A. Erlich, and N. Arnheim. 1985. Enzymatic amplification of β -globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. *Science* **230**:1350–1354.
- Stevenson, M., T. L. Stanwick, M. P. Dempsey, and C. A. Lamonica. 1990. HIV-1 replication is controlled at the level of T cell activation and proviral integration. *EMBO J.* **9**:1551–1560.
- Tax, W. J. M., H. W. Willems, P. P. M. Reekers, P. J. A. Capel,

- and **R. A. P. Koene**. 1983. Polymorphism in mitogenic effect of IgG₁ monoclonal antibodies against T3 antigen on human T cells. *Nature (London)* **304**:445–447.
29. **Thomas, E. K., J. N. Weber, J. McClure, P. R. Clapham, M. C. Singhal, M. K. Shriver, and R. A. Weiss**. 1988. Neutralizing monoclonal antibodies to the AIDS virus. *AIDS* **2**:25–29.
30. **Thompson, P. A., J. S. Gutkind, K. C. Robbins, J. A. Ledbetter, and J. B. Bolen**. Identification of distinct populations of PI-3 kinase activity following T-cell activation. *Oncogene*, in press.
31. **Thompson, P. A., J. A. Ledbetter, U. R. Rapp, and J. B. Bolen**. 1991. The Raf-1 serine-threonine kinase is a substrate for the pp56^{lck} protein tyrosine kinase in human T-cells. *Cell Growth Differ.* **2**:609–617.
32. **Tong-Starkesen, S. E., P. A. Luciw, and B. M. Peterlin**. 1989. Signaling through T lymphocyte surface proteins, TCR/CD3 and CD28, activates the HIV-1 long terminal repeat. *J. Immunol.* **142**:702–707.
- 32a. **Travis, B., and S.-L. Hu**. Unpublished data.
33. **Watson, A. J., J. Klaniecki, and C. V. Hanson**. 1990. Psoralen/UV inactivation of HIV-1-infected cells for use in cytologic and immunologic procedures. *AIDS Res. Hum. Retroviruses* **6**:503–513.
34. **Zack, J. A., S. J. Arrigo, S. R. Weitsman, A. S. Go, A. Haislip, and I. S. Y. Chen**. 1990. HIV-1 entry into quiescent primary lymphocytes: molecular analysis reveals a labile, latent viral structure. *Cell* **61**:213–222.
35. **Zarling, J. M., P. A. Moran, O. Haffar, J. Sias, D. D. Richman, C. A. Spina, D. E. Myers, V. Kuebelbeck, J. A. Ledbetter, and F. M. Uckun**. 1990. Inhibition of HIV replication by pokeweed antiviral protein targeted to CD4⁺ cells by monoclonal antibodies. *Nature (London)* **347**:92–95.