Inhibition of Human Immunodeficiency Virus Type 1 Replication prior to Reverse Transcription by Influenza Virus Stimulation

LIGIA A. PINTO,¹ VESNA BLAZEVIC,¹ BRUCE K. PATTERSON,² C. MAC TRUBEY,³
MATTHEW J. DOLAN,⁴ AND GENE M. SHEARER^{1*}

*Experimental Immunology Branch, National Cancer Institute, National Institutes of Health, Bethesda,¹
and Intramural Research Support Program, SAIC Frederick, NCI-FCRDC, Frederick,³ Maryland;
Laboratory of Viral Pathogenesis, Northwestern University Medical School, Chicago, Illinois²;
and Wilford Hall Medical Center, Lackland AFB, Texas⁴*

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It is now recognized that, in addition to drug-mediated therapies against human immunodeficiency virus type 1 (HIV-1), the immune system can exert antiviral effects via CD8⁺ T-cell-generated anti-HIV factors. This study demonstrates that (i) supernatants from peripheral blood mononuclear cells (PBMC) stimulated with influenza A virus inhibit replication of CCR5- and CXCR4-tropic HIV-1 isolates prior to reverse transcription; (ii) the HIV-suppressive supernatants can be generated by CD4- or CD8-depleted PBMC; (iii) this anti-HIV activity is partially due to alpha interferon (IFN- α), but not to IFN- γ , IFN- β , the β -chemokines MIP-1 α , MIP-1 β , and RANTES, or interleukin-16; (iv) the anti-HIV activity is generated equally well by PBMC cultured with either infectious or UV-inactivated influenza A virus; and (v) the antiviral activity can be generated by influenza A-stimulated PBMC from HIV-infected individuals. These findings represent a novel mechanism for inhibition of HIV-1 replication that differs from the previously described CD8 anti-HIV factors (MIP-1 α , MIP-1 β , RANTES, and CD8 antiviral factor).

Due to the escape by human immunodeficiency virus (HIV) mutants from the therapeutic benefits of highly active, antiretroviral therapy (35, 40), additional or alternate immune-based strategies such as antiviral factors produced by CD8 cells are being considered. These include β -chemokines (8), as well as the CD8 antiviral factor (CAF) (18, 36, 37), first reported more than a decade ago, and the undefined factor(s) generated by alloantigen-stimulated T cells (6, 25). The β -chemokines MIP-1 α , MIP-1 β , and RANTES are limited in their therapeutic potential in that they block CCR5- but not CXCR4-tropic HIV-1 isolates (1, 15). In contrast, the alloantigen-stimulated cells and the factor(s) they produce inhibit viruses that use either or both coreceptors (25). A factor that can inhibit HIV-1 isolates that use different coreceptors becomes important when one is considering the potential clinical value of naturally produced antiviral factors, because changes in coreceptor usage have been noted during disease progression (9).

Influenza A virus is a segmented RNA virus that is endemic throughout the world (10). Immunization of millions of people with different preparations of influenza virus vaccines have been shown to be safe, and the vaccine is routinely administered annually, even to HIV-infected (HIV⁺) patients. The present study demonstrates the generation of an influenza A virus-stimulated anti-HIV activity and tests whether in vitro stimulation with infectious, UV-inactivated virus or the current influenza virus vaccine will elicit the production of an anti-HIV factor(s). This report also analyzes the inhibitory effects of influenza A virus-stimulated supernatants on different HIV-1 isolates; the point of inhibition in the viral replication cycle; the T-cell subsets that produce the factor(s); whether the factor is

a β -chemokine, gamma interferon (IFN- γ), interleukin-16 (IL-16), or IFN- α ; and whether influenza A virus-stimulated peripheral blood mononuclear cells (PBMC) from HIV⁺ patients can generate this anti-HIV activity.

MATERIALS AND METHODS

Influenza virus stimulation of PBMC. Mononuclear cells were isolated by density gradient centrifugation from peripheral blood of healthy HIV-seronegative (HIV⁻) blood donors accrued by the NIH Blood Transfusion Department, as previously reported (25). The two HIV⁺ patients used in the study were from the Wilford Hall Medical Center, Lackland AFB, Texas, and the voluntary, fully informed consent of the patients used in this research was obtained as required by Air Force Regulation 169-9. Blood collection was performed using institutional review board-approved protocols from both institutions.

PBMC (3×10^6 cells/ml) were stimulated in vitro with live, UV-inactivated influenza virus (A/Bangkok/RX73 and A/Puerto Rico/8/34 strains; 1:800) or with the 1998–1999 formula of influenza virus vaccine (1:5,000; Wyeth Laboratories Inc., Marietta, Pa.). The influenza virus vaccine is an inactivated trivalent subunit formulation that contains the hemagglutinin antigens of influenza A H1N1, influenza A H1N3, and influenza B virus strains (each at 30 μ g/ml). PBMC cultured in the absence of stimulation were used as controls in each experiment. In some experiments, PBMC were stimulated with immobilized anti-CD3 monoclonal antibody (10 μ g/ml; Ortho Biotech, Raritan, N.J.) or tetanus toxoid (1:800; Connaught Laboratories, Swiftwater, Pa.). Cell-free supernatants were collected 7 days after culture and frozen at -70°C . Their anti-HIV activity was tested on in vitro HIV-1-infected phytohemagglutinin-stimulated T-cell blasts (PHA blasts) that were generated as previously reported (25). The final concentration of supernatant used in all experiments was 50% (vol/vol).

In some experiments, PBMC were depleted of CD4⁺ or CD8⁺ T cells using anti-CD4 or anti-CD8 immunomagnetic beads (Dynal, Lake Success, N.Y.). After depletion, PBMC contained <6% of the depleted T-cell subset, determined by flow cytometry.

Anti-HIV assay. PHA blasts were infected with HIV-1_{BZ167} (172 50% tissue culture infective doses [TCID₅₀]/10⁵ cells) or HIV-1_{Ba-L} (570 TCID₅₀/10⁵ cells). HIV-1_{BZ167} was grown in human PHA blasts (41). HIV-1_{Ba-L} was grown in monocyte-derived macrophages (23). The HIV-infected PHA blasts (10⁵ cells/100 μ l) were cocultured with supernatants (100 μ l) derived from unstimulated (control) or influenza A virus-stimulated cultures in RPMI 1640 medium (Life Technologies, Gaithersburg, Md.) supplemented with 10% fetal calf serum (Life Technologies) and 10 U of IL-2 (Boehringer Mannheim, Indianapolis, Ind.) per ml in flat-bottom 96-well plates (Costar, Cambridge, Mass.). Supernatants of these cultures were collected 3 and 6 days postinfection, frozen at -20°C , and

* Corresponding author. Mailing address: Experimental Immunology Branch, National Institutes of Health, NCI, Bldg. 10, Rm. 4B36, Bethesda, MD 20892. Phone: (301) 402-3246. Fax: (301) 402-3643. E-mail: shearerg@exchange.nih.gov.

FIG. 1. Inhibition of HIV-1 replication by a culture supernatant from influenza A virus-stimulated PBMC of an HIV⁻ donor. (A) PHA blasts infected with HIV-1_{BZ167} (172 TCID₅₀/10⁵ cells); (B) PHA blasts infected with HIV-1_{Ba-L} (570 TCID₅₀/10⁵ cells); (C) 2-day culture of HIV-1_{IIIB} chronically infected H9 cell line (H9/IIIB). HIV-1 p24 antigen was determined by ELISA. The data (mean $\pm$ SEM) shown are representative of 19 individual experiments performed with HIV-1_{BZ167}-infected, 17 performed with HIV-1_{Ba-L}-infected, and 3 performed with HIV-1_{IIIB}-infected H9 cells, each performed in triplicate.

tested for p24 antigen levels (Coulter p24 enzyme-linked immunosorbent assay [ELISA], Westbrook, Maine). In some experiments, supernatants derived from unstimulated (control) and influenza A virus-stimulated PBMC (100 μ l) were incubated for 2 days with an HIV-1 chronically infected cell line (2×10^4 cells/100 μ l) (H9/HTLV-III NIH 1984, AIDS Research and Reference Reagent Program, Rockville, Md.) in flat-bottom 96-well plates (Costar). Supernatants of cultures were harvested and assayed for HIV-1 p24 antigen (Coulter).

Quantitation of cytokine production after in vitro influenza A virus stimulation. Cytokine (IFN- γ , IL-2, IL-10, tumor necrosis factor alpha [TNF- α], MIP-1 α , MIP-1 β , RANTES [R&D, Cambridge, Mass.], IFN- α , IFN- β , and IL-16 [Biosource International, Camarillo, Calif.]) levels from the culture supernatants were determined by ELISA. Cytokine neutralization experiments of influenza A virus-stimulated supernatants were performed using anti-MIP-1 α , anti-MIP-1 β , and anti-RANTES neutralizing antibodies (50 μ g/ml; R&D), anti-IFN- γ (25 μ g/ml; R&D), anti-IFN- α (100 μ g/ml; Endogen, Woburn, Mass.), and anti-IL-16 (20 μ g/ml; Pharmingen, San Diego, Calif.) antibodies, and equivalent concentrations of goat or mouse isotype control antibodies (R&D).

Cell surface analysis of CD4, CXCR4, and CCR5. Cellular surface expression of CD4, CXCR4, and CCR5 was performed by flow cytometry. T-cell blasts incubated in the presence of unstimulated (control) or influenza A virus-stimulated supernatants for 3 days were harvested and stained with anti-CD4, anti-CXCR4, anti-CCR5 (Pharmingen, San Diego, Calif.), or control monoclonal antibodies directly conjugated with phycoerythrin (PE) for 30 min in the dark, at 4°C, in phosphate-buffered saline containing 1% bovine serum albumin and 0.1% NaN₃. Cells were then washed three times with buffer and analyzed by fluorescence-activated cell sorter (Becton Dickinson). Results are expressed as percent positive cells and mean fluorescence intensity.

HIV DNA quantification. Quantitative, real-time DNA PCR was performed by adding 45 μ l of reaction mix (1 $\times$ Taqman PCR buffer [PE Applied Biosystems, Foster City, Calif.], 4.0 mM MgCl₂, 200 μ M dATP, 200 μ M dCTP, 200 μ M dGTP, 200 μ M dTTP, 200 nM upstream primer, 200 nM downstream primer, 100 nM fluorogenic probe labeled at the 5' end with FAM [5-carboxyfluorescein] and at the 3' end with TAMRA [5-carboxy-tetramethylrhodamine], 10 U of AmpliTaq Gold polymerase) to approximately 500 ng of DNA in 5 μ l of water. Thermal amplification was performed using the following linked profile: 10 min at 95°C, 40 cycles of denaturation (95°C for 15 s), and annealing/extension (60°C for 1 min) in a 7700 sequence detection system (PE Applied Biosystems). Duplicate standard curves with controls for HIV DNA copy number ranging from 10 copies to 10⁵ copies were run with each optical 96-well plate (PE Applied Biosystems). In addition, controls lacking template were included with each plate. Primers and probe sequences used have been previously described (5, 34).

RESULTS

Inhibition of HIV-1 replication by supernatants from influenza A virus-stimulated cells. The effect of supernatants from PBMC cultures unstimulated (control) or stimulated with influenza A virus was tested in HIV-1-infected PHA blasts and in an H9 T-cell line chronically infected with HIV-1_{IIIB}. The supernatant of infectious influenza A virus-stimulated PBMC from a healthy HIV-seronegative (HIV⁻) donor inhibited HIV-1 replication in PHA blasts infected with either the BZ167 isolate (which preferentially uses CXCR4 and CCR3 coreceptors [25]) (Fig. 1A) or the Ba-L isolate (which uses CCR5 coreceptor [8]) (Fig. 1B). Inhibition of HIV replication was also seen in the HIV-1_{IIIB} chronically infected H9 cell line

(Fig. 1C). The extents of inhibition were 94, 96, and 85%, respectively, and the example shown is representative of multiple experiments. In contrast, supernatants derived from anti-CD3- or tetanus toxoid-stimulated PBMC of six donors did not appreciably inhibit HIV-1_{BZ167} replication (33% $\pm$ 8% [mean $\pm$ standard error of the mean {SEM}] and 35% $\pm$ 12% inhibition, respectively). This result demonstrated that not all forms of stimulation induce significant generation of HIV-suppressive activity. The inhibitory effect of influenza A virus-stimulated supernatants on HIV-1_{BZ167} and HIV-1_{Ba-L} replication was dose dependent (Fig. 2) and demonstrated a considerable effect (62 and 77% inhibition of replication, respectively) when present at a concentration of 12.5% (vol/vol).

To assess possible toxic effects due to exposure of HIV-infected PHA blasts to influenza A virus-stimulated supernatants, we assessed the viability of cultures by trypan blue exclusion. No significant difference in the viability of HIV-1-infected PHA blasts with or without influenza A virus-stimulated supernatants was observed (data not shown). In addition, influenza A virus itself (1:800 dilution) did not significantly affect HIV-1 replication in PHA blasts (data not shown).

Influenza A virus-stimulated anti-HIV activity is not due to IFN- γ , β -chemokines, IFN- β , or IL-16 but is partially blocked by anti-IFN- α antibodies. We found that influenza A virus-stimulated supernatants contained IFN- γ , the β -chemokines

FIG. 2. Dose-response curves of the effect of influenza A virus-stimulated supernatants on HIV-1_{BZ167} (A) and HIV-1_{Ba-L} (B) replication. PHA blasts were infected with HIV-1_{BZ167} (172 TCID₅₀/10⁵ cells) or HIV-1_{Ba-L} (570 TCID₅₀/10⁵ cells) and cultured with different concentrations (volume/volume) of supernatants derived from PBMC stimulated with live influenza A virus (circles) or unstimulated (open squares). p24 antigen production was assayed at 3 days postinfection by ELISA. The results are expressed as mean $\pm$ SEM of four independent experiments performed in triplicate.

TABLE 1. Cytokine levels in supernatants from PBMC stimulated with influenza virus

Stimulus	Cytokine content (ng/ml) ^a						
	IFN- γ (23 ^b)	MIP-1 α (6)	MIP-1 β (6)	RANTES (6)	IFN- α (11)	IL-16 (5)	IFN- β (5)
Control	0.007 $\pm$ 0.003	0.13 $\pm$ 0.08	0.17 $\pm$ 0.09	0.48 $\pm$ 0.14	0.013 $\pm$ 0.009	0.31 $\pm$ 0.06	<10
Influenza virus	0.55 $\pm$ 0.13	2.9 $\pm$ 2.4	0.58 $\pm$ 0.23	4.90 $\pm$ 3.44	2.21 $\pm$ 0.32	0.49 $\pm$ 0.05	<10

^a PBMC were cultured in the absence (control) or presence of live virus as described in Materials and Methods. Cytokine levels were determined by ELISA and are expressed as mean $\pm$ SEM.

^b Number of supernatants analyzed.

MIP-1 α , MIP-1 β , and RANTES, IFN- α , and IL-16 but undetectable levels of IFN- β (Table 1). To test whether the influenza A virus-stimulated inhibitory activity was due to IFN- γ , β -chemokines (MIP-1 α , MIP-1 β , and RANTES), IFN- α , or IL-16, which have been reported to inhibit HIV-1 replication (2, 4, 8, 15, 38, 39), PBMC from HIV⁻ donors were stimulated with infectious influenza A virus. The supernatant generated was cocultured with PHA blasts infected with HIV-1_{BZ167} or HIV-1_{Ba-L} in the presence or absence of a neutralizing anti-IFN- γ monoclonal antibody, a pool of neutralizing antibodies against MIP-1 α , MIP-1 β , and RANTES, polyclonal anti-IFN- α , anti-IL-16, or negative control antibodies. Anti-IFN- γ or β -chemokine antibodies did not affect the HIV-suppressive activity of influenza A virus-stimulated supernatants (Fig. 3A and B). Similarly, an anti-IL-16 antibody did not inhibit the HIV-suppressive activity of the influenza A virus-stimulated supernatant (83 versus 81% inhibition of HIV-1_{BZ167} replication by a influenza A virus-stimulated supernatant in the presence of an isotype control or anti-IL-16 antibody, respectively). However, anti-IFN- α antibody partially (30 to 45%) reversed the inhibitory effect induced by influenza A virus-stimulated supernatants in both HIV-1_{BZ167} and HIV-1_{Ba-L} replication (Fig. 3C and D). The blocking activity of the antibodies against IFN- γ , β -chemokines, IFN- α , or IL-16 was verified by measuring these cytokines by ELISA after neutralization. Furthermore, no significant correlation ($r = 0.043$ to 0.304) was found between the levels of IL-2, IFN- γ , TNF- α , IL-10, MIP-1 α , MIP-1 β , and RANTES in the influenza A virus-stimulated

supernatants and the antiviral activity observed (data not shown).

T-cell subset analysis for generation of anti-HIV activity. To determine whether both CD4⁺ and CD8⁺ T cells are required for generation of the anti-HIV activity observed and/or whether the factor(s) can be produced by only one T-cell subset, PBMC from HIV⁻ donors were either unfractionated or depleted of each subset. The data in Table 2 demonstrate that the antiviral activity stimulated by influenza A virus can be generated by PBMC preparations depleted of either CD4⁺ or CD8⁺ T cells (in five of nine donors). However, two donors (6 and 7) exhibited appreciable loss of anti-HIV activity by CD8 but not CD4 depletion, and two others (8 and 9) exhibited appreciable loss of anti-HIV activity by CD4 but not CD8 depletion. The antiviral activity by CD4 or CD8 T-cell-depleted culture supernatants was only partially blocked by anti-IFN- α antibodies, similar to that observed in unfractionated cultures (data not shown).

Anti-HIV activity generation by live and inactivated influenza A virus and influenza virus vaccine. We tested whether the generation of the antiviral activity by influenza A virus-stimulated PBMC required infectious influenza A virus or whether UV-inactivated virus or the 1998–1999 stock of influenza virus vaccine would also generate anti-HIV activity. The UV-inactivated influenza A virus (which enters leukocytes but does not replicate [26]) was as effective as infectious influenza A virus (15 of 17 and 28 of 31 donors produced supernatants with >50% inhibition of HIV replication for UV-inactivated

FIG. 3. Test of inhibition of influenza A virus (Flu)-stimulated anti-HIV activity on HIV-1_{BZ167} (A and C) or HIV-1_{Ba-L} (B and D) infected PHA blasts, using antibodies (Abs) specific for IFN- γ or a pool of antibodies against MIP-1 α , MIP-1 β , and RANTES (A and B) or for IFN- α (C and D). The blocking activity of the anti-IFN- α antibody reduced the level of IFN- α in the supernatant from 4.62 ng/ml to 0.002 ng/ml. The supernatant used in panels A and B contained 0.38 ng of IFN- γ , 2.6 ng of RANTES, 0.66 ng of MIP-1 α , and 0.26 ng of MIP-1 β per ml. The neutralizing antibodies against anti-IFN- γ reduced the levels of IFN- γ to 0.044 ng/ml. The anti- β -chemokine antibodies reduced the β -chemokine levels by 80 to 90%. The results, expressed as mean $\pm$ SEM, are representative of three experiments performed in triplicate.

FIG. 4. Comparison of anti-HIV activities by supernatants from PBMC of HIV⁺ donors stimulated with infectious influenza A virus ($n = 31$ donors), UV-inactivated influenza A virus ($n = 17$), or an influenza virus vaccine ($n = 13$) on PHA blasts infected with HIV-1_{BZ167}. The horizontal lines represent mean values.

and live influenza A virus, respectively) (Fig. 4). The mean percent inhibition of supernatants from HIV⁺ donors stimulated with influenza virus vaccine was approximately half of that of the infectious and inactivated virus-stimulated cultures. However, the data obtained from the 13 individuals tested could be divided into two categories: five that strongly inhibited HIV replication when their PBMC were exposed to the influenza virus vaccine (55 to 89% inhibition of HIV-1_{BZ167} replication) and eight that did not (0 to 35% inhibition of HIV replication). Vaccine-stimulated anti-HIV activity did not correlate with influenza A virus-specific T-helper responses ($r = 0.1$) in a subset of eight individuals analyzed (data not shown). The antiviral activity described here appears to be selective for HIV infection, because influenza A virus infection of human T-cell blasts determined by intracellular immunofluorescence staining using anti-influenza virus nucleoprotein antibody (11, 27) was not affected by the influenza A virus-stimulated anti-HIV supernatants (data not shown).

TABLE 2. Effects of supernatants derived from influenza virus-stimulated CD4-depleted or CD8-depleted PBMC on HIV-1_{BZ167} replication^a

HIV ⁺ donor no.	% Inhibition of HIV-1 _{BZ167} replication		
	PBMC	CD4-depleted PBMC	CD8-depleted PBMC
1	94	90	83
2	87	88	99
3	83	70	90
4	95	63	83
5	77	79	84
6	66	75	20
7	75	80	30
8	94	42	97
9	89	27	75

^a PBMC depletions were performed as described in Materials and Methods.

Influenza A virus-stimulated inhibition of HIV-1 early and late reverse transcripts. HIV-1_{BZ167}-infected blasts cultured in the presence of influenza A virus-stimulated supernatants were analyzed by quantitative, real-time DNA PCR with primers specific for late (long terminal repeat [LTR]-*gag*) and early (LTR U3/R) reverse transcripts. Both late (Fig. 5A) and early (Fig. 5B) reverse transcripts were significantly decreased by influenza A virus-stimulated supernatants (80 and 94% inhibition, respectively), demonstrating that influenza A virus-stimulated anti-HIV activity occurs prior to reverse transcription. In contrast, no appreciable changes in CCR5 and CXCR4 mRNA coreceptor expression were detected by reverse transcription-PCR in infected cells exposed to influenza A virus-stimulated supernatants (data not shown). The results in Fig. 5C verify that the influenza A virus-stimulated supernatant used had a strong inhibitory effect on HIV-1 replication (99% inhibition), as measured by p24 antigen production.

Influenza A virus-stimulated supernatants do not inhibit cell surface expression of CD4, CXCR4, or CCR5. To further address the mechanism of inhibition of HIV-1 replication by influenza A virus-stimulated supernatants, we tested the effect

FIG. 5. Demonstration that inhibition of HIV-1 replication by influenza A virus-stimulated supernatants occurs prior to reverse transcription. Levels of late (LTR-*gag*) (A) and early (LTR U3/R) transcripts (B) in HIV-1_{BZ167}-infected blasts were determined by quantitative, real-time DNA PCR. Results represent means (number of copies of reverse transcripts/50,000 ± 10,000 cells) ± standard deviation of two independent experiments. HIV-1 p24 antigen production by HIV-1_{BZ167}-infected PHA blasts (C) was determined by ELISA.

FIG. 6. Effect of influenza A virus-stimulated supernatants on the surface expression of CD4, CXCR4, and CCR5 in T-cell blasts. PHA blasts were incubated with supernatants derived from unstimulated (Control) or influenza A virus (Flu)-stimulated PBMC for 3 days. Cell surface staining was performed by flow cytometry as indicated in Materials and Methods. Ab, antibody.

of these supernatants on the cell surface expression of CD4, CXCR4, and CCR5 in PHA blasts. We observed that influenza A virus-stimulated supernatants did not alter the cell surface expression of CD4, CXCR4 or CCR5 (Fig. 6) compared to control supernatants derived from unstimulated PBMC (Table 3).

Influenza A virus-stimulated anti-HIV activity in HIV-infected individuals. As shown above (Table 2), the generation of influenza A virus-stimulated anti-HIV activity can be mediated by CD4⁺ or CD8⁺ T cells and does not require the presence of both T-cell subsets. The possibility that influenza A virus-stimulation of PBMC from HIV⁺ patients, even those who are unable to elicit T-cell proliferative or IFN- γ responses to influenza A virus, could generate anti-HIV activity was tested in two HIV⁺ patients (Fig. 7). The percentages of inhibition of HIV replication were similar in the PBMC from two healthy control donors and two HIV⁺ patients (Fig. 7A), despite a wide range in the proliferative responses to infectious influenza A virus (stimulation index range of 3 to 55) (Fig. 7B) and different IFN- γ or IFN- α production patterns (Fig. 7C).

DISCUSSION

The present study demonstrates that influenza A virus-stimulated PBMC produce a soluble factor(s) that inhibits HIV-1 replication. The anti-HIV activity observed differs from those found for the β -chemokines and CAF, the two most extensively

studied anti-HIV factors, in several ways. First, the β -chemokines MIP-1 α , MIP-1 β , and RANTES block CCR5- but not CXCR4-tropic viral isolates (1, 15). In contrast, the influenza A virus-stimulated factor(s) inhibits both a CCR5-tropic isolate (Ba-L) (8) and a dualtropic isolate that predominantly uses CXCR4 and CCR3 (BZ167). Second, the fact that CAF is produced exclusively by CD8⁺ T cells (18) contrasts with the factor(s) generated by influenza A virus stimulation, which can be generated by non-CD8⁺ T cells. Third, CAF blocks HIV replication at the level of transcription (13, 20), which occurs later than a point in the viral replication at which the influenza A virus-stimulated activity functions. These comparisons with the β -chemokines and CAF raise the possibility that influenza A virus stimulation of different subsets of T cells and possibly non-T cells generates anti-HIV activity through a potential novel mechanism.

To examine the potential role of β -chemokines, IFN- γ , IFN- α , or IL-16 in the inhibition of viral replication by influenza A virus-stimulated supernatants, we performed blocking experiments using neutralizing antibodies against these cytokines. Addition of anti- β -chemokine, anti-IFN- γ , or anti-IL-16 antibodies to influenza A virus-stimulated supernatants did not reduce their HIV-inhibitory effect, which further suggests that the antiviral activity observed is not directly due to these cytokines. In addition, the lack of correlation found between the levels of these chemokines as well as IL-2, IFN- γ , TNF- α , and IL-10, and the antiviral activity observed by influenza A virus-stimulated supernatants further indicates that these cytokines are not major direct mediators of the antiviral activity detected. However, the HIV-inhibitory effect was partially (up to 45%) blocked by the presence of anti-IFN- α antibodies, which indicates that this cytokine is an important mediator of the antiviral activity detected against dualtropic and monocyte-tropic strains of HIV. These findings are in agreement with previous reports demonstrating that IFN- α inhibits HIV replication in vitro (30–32, 39). Since endogenous IFN- α has been shown to be more effective at blocking HIV replication in vitro than recombinant IFN- α , it is possible that stimulation of endogenous IFN- α production may offer some potential advantages over clinical protocols using exogenous administration of recombinant IFN- α (16, 21, 29).

TABLE 3. Effect of influenza virus-stimulated supernatants in CD4, CCR5, and CXCR4 cell surface expression^a

Supernatant	CD4	CXCR4	CCR5
Control	48 $\pm$ 2	78 $\pm$ 2	15 $\pm$ 2
Influenza virus stimulated	50 $\pm$ 2	75 $\pm$ 1	15 $\pm$ 1

^a PHA blasts were incubated for 3 days with supernatants derived from PBMC unstimulated (control) or stimulated with live influenza A virus and then stained for CD4, CXCR4, and CCR5. Results were determined by flow cytometry as described in Materials and Methods and are expressed as percentage of positive cells (CD4) and mean fluorescence intensity of staining with PE-anti-CXCR4 or CCR5 corrected for nonspecific staining with PE-conjugated isotype control (CXCR4 and CCR5). The results represent mean $\pm$ SEM of three different experiments.

FIG. 7. Comparison of PBMC from two HIV⁻ donors (1 and 2) and two HIV⁺ donors (3 and 4) for infectious influenza A virus-stimulated inhibition of HIV-1_{BZ167} replication (A), T-cell proliferation (B), and IFN-γ and IFN-α production (C). Both patients were asymptomatic, with CD4 T-cell counts of 699 (donor 3) and 465 (donor 4) cells/μl. T-cell proliferation results are expressed as stimulation index (S.I.), calculated as counts per minute of cultures in the presence of influenza A virus/counts per minute of cultures with medium alone. IFN-γ and IFN-α production was measured by ELISA.

The fact that the inhibition of HIV replication was not completely blocked by the anti-IFN-α antibodies, even in CD4- and CD8-depleted cultures, suggests that other antiviral factors or possible synergistic cytokine effects may account for the remaining antiviral activity. Our observation that another factor may contribute to the anti-HIV effect is consistent with the poor relationship between IFN-α levels and inhibition of HIV-1 replication in Fig. 7. Studies are in progress to identify the non-IFN-α component(s) of this anti-HIV activity and to fully characterize the molecular mechanism of inhibition of viral replication.

The influenza A virus-stimulated production of anti-HIV activity appears to be independent of the requirement for both CD4⁺ and CD8⁺ T cells. The results of the cell depletion experiments are complicated by the observation that two of nine donors generated anti-HIV activity in CD4-depleted but not CD8-depleted cultures. Additional experiments will be required to identify the various T-cell and possibly non-T-cell subsets that can generate the anti-HIV activity. Nevertheless, it is clear that the production of the anti-HIV factor(s) is not limited to CD8⁺ T cells, in contrast to CAF (18).

The fact that CD4⁺-CD8⁺ T-cell collaboration at a functional level is not required for generation of the anti-HIV activity raises the possibility that HIV⁺ patients who have low CD4⁺ T-cell counts could generate this anti-HIV factor(s). This suggestion is supported by our preliminary findings that both of two HIV⁺ patients tested thus far generated anti-HIV activity when stimulated with live influenza A virus. In addition, no correlation of anti-HIV activity with influenza A virus-stimulated T-cell proliferation or IFN-γ production was observed in either HIV⁺ or HIV⁻ donors. These observations raise the possibility that T cells from HIV⁺ patients can respond to live influenza A virus stimulation by producing the anti-HIV factor(s) that would be independent of a strong memory T-cell response to the virus.

The reports about the effect of influenza virus vaccine administration on HIV-1 load in HIV-infected patients are contradictory. Some studies have described transient increases in viral load (22, 33), while others have reported no effect in HIV⁺ patients who were on antiretroviral therapy at the time of vaccination (12, 14). Analysis of anti-HIV activity generated by three different types of influenza A virus stimulation (live virus, inactivated virus, and influenza virus vaccine) demonstrates that UV-inactivated influenza A virus, but not influenza virus vaccine, induces anti-HIV activity similar to that induced by live influenza A virus. Although the mechanisms underlying these findings have not been identified, it is possible that the

reduced anti-HIV activity of the influenza virus vaccine is due to expression of different influenza virus antigens. Alternatively, this difference could be related to distinct pathways of antigen processing and presentation (24), which may activate different arms of the immune system.

Previous studies of the effects of CD8 antiviral factors on HIV-1 reverse transcription and viral transcription have shown inhibition of HIV replication postintegration, at the level of transcription (13, 20). In the present study, however, the block in the HIV-1 replication occurs prior to reverse transcription because the levels of both late (LTR-gag) and early (LTR U3/R) reverse transcripts were significantly decreased by influenza A virus-stimulated supernatants. These results suggest that the antiviral activity mediated by influenza A virus-stimulated supernatants is different from the antiviral activity produced by CAF, since CAF does not affect the levels of early or late reverse transcripts (5). The possibility that influenza A virus-stimulated supernatants could exert their anti-HIV effect via down-modulation of surface expression of CD4 or the HIV coreceptors CXCR4 or CCR5 is unlikely since influenza A virus-stimulated supernatants did not inhibit the expression of these receptors in T-cell blasts.

Although this is the first demonstration of induction of in vitro anti-HIV activity by influenza A virus, suppression of viral replication by unrelated viruses has been previously reported. In fact, infection with human herpesvirus 6 can suppress HIV replication in CD4⁺ T cells (17) and dendritic cells (3), and cytomegalovirus has been shown to inhibit replication of hepatitis B virus replication (7). It should be noted in this context that immunization with virus-modified tumor cells has been proposed as an immune-based therapeutic strategy for cancer (19, 28).

Our in vitro studies do not necessarily imply that infection with influenza virus should be used as an immune-based therapeutic approach. However, the identification of the stimuli and mechanisms involved in the elicitation of influenza A virus-induced antiviral immunity may contribute to the design of novel, safe, complementary anti-HIV therapeutic strategies.

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