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Research Article

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Polyl · polyC$_{12}$U–Mediated Inhibition of Loss of Alloantigen Responsiveness and Viral Replication in Human CD4+ T Cell Clones Exposed to Human Immunodeficiency Virus In Vitro

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Abstract

Two alloreactive human CD4+ T cell clones, recognizing HLA-DR2 and HLA-DR1 determinants, lost their specific proliferative capacity after infection with HIV. This system was used to explore the effect of polyI · polyC$_{12}$U on HIV replication and immune suppression. The mismatched double-stranded RNA blocked HIV-associated particulate reverse transcriptase activity and viral-mediated cytopathic effects. Also, polyI · polyC$_{12}$U preserved the alloreactivity of T cell clones after exposure to HIV. PolyI · polyC$_{12}$U appeared to act at a level subsequent to host cell infection and reverse transcription. It had no effect on the enhancement of gene expression by the HIV transcription unit *tat*$_{III}$.

These findings indicate that early in the course of infection of CD4+ T lymphocytes, HIV can directly abrogate proliferation to specific allodeterminants, and that this function is preserved in the presence of polyI · polyC$_{12}$U. They also provide insight into the mechanism of antiviral action of a class of agent with potential clinical utility in AIDS.

Introduction

Immunologic abnormalities characteristic of infection with HIV, the etiologic agent of the AIDS, appear secondary to quantitative and qualitative defects in the CD4+ helper–inducer subset of T lymphocytes (1, 2). Mononuclear cells of AIDS and AIDS-related complex (ARC)[1] patients have an intrinsic defect in the ability to recognize and respond to soluble antigen (1), self-antigen (3, 4), and alloantigen (4, 5). The cell subpopulations responsible for these perturbations are often not clearly defined. Antigen-presenting cells as well as T lymphocytes may be infected with HIV and their functions consequently altered. Human T lymphotropic virus type I (HTLV-I), another human retrovirus tropic for T cells, can disrupt the alloreactivity of CD4+ T cell clones, allowing them to proliferate to multiple class II HLA determinants (6). HTLV-I can also infect and alter the cytolytic activity of CD8+ T cells (7).

We have used two CD4+ human T cell clones, one with HLA-DR2 specificity, A57 (8), and another recognizing HLA-DR-1, 86 (formerly referred to as TCL 1–8 [9]), to examine the effect of HIV on a discrete immune function. This assay also provided a model system to explore the mechanism of an agent with antiviral activity. The ability of IFN inducers to disrupt several steps in viral life cycles, including translation and maturation, make them attractive candidates for examination in HIV infection (10). PolyI · polyC$_{12}$U was selected for particular attention as, like most double-stranded RNAs, it induces IFNs, it may act synergistically with IFNs, and it has a history of use in animal and human trials (11).

In this report we demonstrate that HIV infection of CD4+ T cell clones blocks their alloproliferation, that treatment of these lymphocytes with polyI · polyC$_{12}$U inhibited HIV replication and preserved their ability to respond to specific class II alloantigens, that polyI · polyC$_{12}$U exerts an effect at a level other than the transcriptional or translational steps related to the HIV *trans*-acting gene *tat*$_{III}$, and that radioimmunoprecipitation of HIV-specific proteins from cells exposed to HIV in the presence of polyI · polyC$_{12}$U revealed synthesis of some viral peptides despite complete inhibition of particulate reverse transcriptase activity.

Methods

Cells. A57 is an Ia+, CD3+, CD4+, CD8− allospecific helper T lymphocyte that proliferates in response to HLA-DR2 positive antigen-presenting cells (8). It provides MHC-restricted polyclonal help for DR2+ B cells, and initiates a B cell proliferative response that is MHC restricted at the inductive level (8). 86 is another alloproliferative helper T cell clone, of identical phenotype, which recognizes HLA-DR1 determinants (9). Both clones are maintained in Iscove's modified Dulbecco's medium (IMDM; Gibco Laboratories, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) and 10% nonrecombinant IL-2 (Electro-Nucleonics, Inc., Fairfield, NJ). Cells are plated at 1×10^5/ml in 2-ml aliquots in 12-chamber cluster plates (Costar 3512; Costar Data Packaging Corp., Cambridge, MA) and maintained at approximately this concentration by replating every 3–4 d. Every 7 d, 0.5×10^6 feeder cells, consisting of E-rosette negative cells of appropriate HLA-DR phenotype, prepared as previously detailed (8, 9), are irradiated (2,000 rad from a ^{137}Cs source) and added to each well.

PBMC were isolated by density gradient centrifugation of heparinized venous blood obtained from HIV seronegative donors. They were stimulated with PHA as described elsewhere (12).

H9, a human CD3+, CD4+ lymphoblastoid T cell line permissive

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1. *Abbreviations used in this paper:* ARC, AIDS-related complex; CAT, chloramphenicol acetyl transferase; FBS, fetal bovine serum; *gag*, group antigen; HTLV-I, human T lymphotropic virus type I; IMDM, Iscove's modified Dulbecco's medium; LTR, long-terminal repeat; MuLV, murine leukemia virus; polyI · C, polyriboinosinic acid–polyribocytidylic acid.

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for the replication of HIV and partially resistant to its cytopathic and cytolytic effects, was infected with a strain of HIV known as HIV_B and used as a source for continuous production of infectious virus (13). These H9–HIV_B cells were grown at a density of 1×10^6/ml in RPMI 1640 (Gibco Laboratories) plus 10% FBS. Cell-free supernatants were collected and pooled at 4-d intervals, filtered through 0.45-μm membranes (Millipore/Continental Water Systems, Bedford, MA), and stored at −70°C. Infectivity assays (13, 14) indicated that 1 ml of HIV_B stock corresponded to an ID_{50} of 1,000.

HIV infection of T cell clones and PBMC. A57 or 86 cells were harvested 7 d subsequent to their last exposure to feeder cells, washed with PBS, and resuspended at 0.5×10^6/ml in IMDM plus 10% FBS plus 10% IL-2. Cells, plated in macrowells (Costar Data Packaging Corp.), were exposed to either 100 or 1,000 ID_{50} of viral stock, the medium completely changed at 18 h, and 0.5×10^6 irradiated feeder cells were added at 48 h. One-half of the medium was removed and replaced with fresh IMDM plus 10% FBS plus 10% IL-2 every 3–4 d thereafter.

PBMC were activated with PHA for 24–72 h, washed with PBS, then resuspended at 2×10^6/ml in RPMI 1640 plus 10% FBS plus 10% IL-2 in polyvinyl flat-bottom macrowells. 1,000 ID_{50} of HIV_B were added, the medium completely changed at 48 h, and one-half of the medium was removed and replaced with fresh RPMI 1640 plus 10% FBS plus 10% IL-2 every 3–4 d subsequently.

Functional analysis of A57 and 86 cells. Cells were collected, washed three times with PBS, and viability assessed by trypan blue dye exclusion (12). 2×10^4 viable cells were resuspended in 0.1 ml of IMDM plus 10% FBS together with an equal volume of medium or medium containing 1×10^5 irradiated antigen-presenting cells carrying either the appropriate (HLA-DR2 and HLA-DR1, respectively) or irrelevant (HLA-DR4/6) allodeterminants. Selected cultures were supplemented with 10% IL-2. All groups were assayed in triplicate. Cells were incubated for 36 h in the absence of IL-2, or for 60 h in the presence of IL-2, in flat-bottom polyvinyl microwells. 18 h before culture termination, cells were pulsed with 0.1 μCi of [^{3}H-methyl]-thymidine (1.9 Ci/mM sp act; New England Nuclear, Boston, MA). The contents of each well were harvested and incorporation of radioactivity was measured by liquid scintillation counting.

PolyI · polyC$_{12}$U treatment and determination of interferon synthesis. PolyI · polyC$_{12}$U was manufactured as previously detailed (11). It was reconstituted with distilled water to give a final salt concentration of 150 mM NaCl, 10 mM PO_4, and 1 mM $MgCl_2$. It was aliquoted and stored at −70°C, and thawed just before use. In the clonal T cell experiments, lymphocytes were incubated with 250 μg/ml polyI · polyC$_{12}$U, diluted in IMDM plus 10% FBS, for 2 h before HIV exposure. In the PBMC infectivity assays, the molecule was added to PHA-activated cells together with virus.

Gamma IFN activity was measured in cell-free culture supernatants by radioimmunoassay (Centocor, Malvern, PA) specific for gamma IFN (15). It was compared with a National Institutes of Health (NIH) standard, and is expressed in NIH units (15).

Alpha IFN levels were determined in cell supernatants by a cytopathic effect-inhibition bioassay that employed GM2767 fibroblasts, vesicular stomatitis virus, and a polyclonal anti-human alpha IFN antibody (16).

Reverse transcriptase activity. Assays were performed as previously described (17). Briefly, 0.75 ml of cell-free culture supernatant was mixed with 0.25 ml of a 30% solution of polyethylene glycol 3400 (Sigma Chemical Co., St. Louis, MO) in water and precipitated at 4°C for 18 h in 1.5-ml polypropylene tubes. Samples were then centrifuged at 10,000 *g* for 3 min. The pellet was resuspended in 25 μl of virus-solubilizing buffer (0.8 M NaCl, 0.5% Triton X-100, 0.5 mM phenylmethylsulfonyl fluoride, 50 mM Tris [pH 7.9], 1 mM dithiothreitol, and 20% glycerol), kept at 4°C for 5 min, and RNA template buffer was added. This consists of 64 mM Tris (pH 7.9), 11 mM $MgCl_2$, 1.1 mM dithiothreitol, 0.14 mM dATP, 5 U of poly(rA) · oligo(dT)$_{12-18}$ (P-L Biochemicals Inc., Milwaukee, WI), and 5 mCi of [^{3}H-methyl]-thymidine triphosphate (20 Ci/mmol sp act; New England Nuclear). The reaction is run for 2 h at 37°C with constant rocking, then stopped with 10% cold trichloroacetate containing 0.1 M sodium pyrophosphate, 1 mM EDTA, and 10 mM Tris (pH 7.9). Precipitates were collected on fiberglass filters presoaked in 5% trichloroacetate, washed with 5% cold trichloroacetate, dried, and counted using liquid scintillant.

Metabolic labeling. Cells were harvested, washed in serum-free RPMI 1640, and resuspended at 5×10^6/ml in cysteine-free RPMI 1640 for 1 h at 37°C. 100 μCi of [^{35}S]cysteine (300 Ci/mmol; New England Nuclear) were added to each ml of cells, and the incubation continued for 4–6 h. A soluble cell lysate was prepared by disruption with lysate buffer (0.15 M NaCl, 0.05 M Tris [pH 7.2], 1% Triton X-100, 1% sodium deoxycholate, and 0.1% SDS) and centrifuged at 10,000 *g* for 5 min. Lysates to be used for radioimmunoprecipitation are cleared once with 10 μl of an HIV seronegative control IgG (2 mg/ml) bound to protein A–Sepharose beads (CL-4B; Sigma Chemical Co.). Portions are then reacted for 16 h at 4°C with 5 μl of IgG (2 mg/ml) obtained from an HIV seropositive patient. All IgG fractions were derived from serum samples using a ZetaChrom ion exchange chromatography disc (CUNO, Inc., Meriden, CT). Immunoprecipitates were eluted from the Sepharose beads in sample buffer containing 0.1 M dithiothreitol, 2% SDS, 0.08 M Tris [pH 6.8], 10% glycerol, and 0.2% bromphenol blue by boiling for 2 min. All samples were analyzed on a 7.5% acrylamide-resolving gel with a 3.5% acrylamide-stacking gel in a discontinuous buffer system (17). Gels were impregnated with scintillator, dried, and radioactive bands were detected by autoradiography (17).

HIV-associated trans-activation. The ability of the tat_{III} transcription unit of HIV to enhance the expression of the chloramphenicol acetyl transferase (CAT) gene when CAT is linked to the long terminal repeat (LTR) of HIV was measured as previously described (18). Briefly, 1×10^6 CD4+ human lymphoblastoid SK cells were washed with serum-free RPMI 1640 and incubated for 1 h at 37°C in 2 ml of serum-free RPMI containing 50 mM Tris (pH 7.3), 250 μg/ml DEAE-dextran (Sigma Chemical Co.), and 10 μg of plasmid DNA. Two plasmids were used either singly (CAT alone) or together (co-transfection of CAT and tat_{III} containing plasmids). These transient expression vectors have been illustrated elsewhere (18). The tat_{III} plasmid pCV-1 contains a duplicated SV40 replication origin, an adenovirus late promoter, splice sites from adenovirus and mouse immunoglobulin genes, mouse dihydrofolate reductase complementary DNA, a SV40 polyadenylation signal, and the *tat* gene. The CAT plasmid pC15CAT contains SV40 regulatory sequences and the LTR and a portion of the 3′-*orf* (open reading frame) of HIV. After transfection, cells were washed with serum-free RPMI 1640 and incubated in 10 ml of RPMI 1640 plus 10% FBS for 48 h at 37°C. Cells were then harvested, washed with PBS, resuspended in 100 μl of 0.25 M Tris (pH 7.8), and cellular extracts were prepared by three cycles of freezing (in ethanol and dry ice) and thawing. CAT activity was determined by incubating 20 μl aliquots of cell extracts with [^{14}C]chloramphenicol (New England Nuclear) and 2.5 mM acetyl coenzyme A (P-L Biochemicals Inc.) at 37°C for 18 h. The acetylated forms of chloramphenicol were separated from the unacetylated form by ascending thin layer chromatography, using Si-HPF plates (7011-4; J. T. Baker Chemical Co., Phillipsburg, NJ) in a chamber containing chloroform and methanol (18). The chromatogram was then autoradiographed.

Treatment of cultures with anti-IFNs. Stock solutions of a sheep anti-human alpha IFN (Interferon Sciences Inc., New Brunswick, NJ), and a monoclonal mouse anti-human gamma IFN (graciously provided by Dr. Berish Rubin, The New York Blood Center, New York, NY) were made so that 1 ml contained 10,000 U of activity (1 U is defined as the amount of reagent required to neutralize 10 U of alpha or gamma IFN to 1 U). These antisera were added to PHA-activated target PBMC cultures simultaneously with stock HIV and polyI · polyC$_{12}$U. Additional antibody was included with each change of medium.

Table I. Effect of HIV on the Alloreactivity of CD4+ Cell Clone A57 in the Presence and Absence of PolyI · polyC$_{12}$U

Group	HIV	ID_{50}	PolyI · polyC$_{12}$U (250 μg/ml)	Mitogenic response of A57 clone*			Stimulation index‡
				Antigen-presenting cell			
				None	HLA-DR4/6	HLA-DR2	
1	−	—	−	204±120	431±15	5,550±280	27.2
2	+	1,000	−	484±87	460±72	700±26	1.45
3	+	100	−	377±54	518±70	729±29	1.93
4	+	1,000	+	349±67	245±38	1,514±151	4.34
5	+	100	+	253±30	220±34	4,551±143	18.0

* A57 cells were infected with HIV in the presence or absence of polyI · polyC$_{12}$U, harvested on day 12, washed, and 1×10^4 viable cells cultured with 1×10^5 irradiated E-rosette negative antigen-presenting cells for 36 h in the absence of IL-2. [^{3}H]Thymidine was added 18 h before culture termination. Each value represents the mean counts per minute (±SD) of triplicate cultures. ‡ Calculated as (mean counts per minute of A57 + HLA-DR2 antigen-presenting cells)/(mean counts per minute of A57 + no antigen-presenting cells).

Results

Effect of HIV on alloproliferation of A57 cells. The allospecificity of clone A57 was reestablished by demonstrating its blastogenic response to irradiated HLA-DR2 but not HLA-DR4/6 antigen-presenting cells in either the absence (Table I) or presence (Table II) of exogenous IL-2. Several other non-DR2 determinants were also examined, and elicited no incorporation of [^{3}H]thymidine into DNA above background levels. Two different concentrations of HIV were capable of abrogating this MHC-restricted proliferation in the absence (Table I) or presence (Table II) of IL-2.

Equal numbers of viable lymphocytes were used in these experiments. HIV-infected A57 cells cultured in the presence of 10% IL-2 gave a minor (two to three times background) DNA synthetic response to a non-DR2 allodeterminant (Table II). This result, not seen in the absence of IL-2 (Table I), was consistent in each of three experiments performed.

The brief incubation period employed in these assays (36 h) makes it unlikely that HIV shed into the culture medium infected the irradiated antigen-presenting cells and thereby diminished alloproliferation. To directly address this concern, 100 ID_{50} of HIV was added to cultures on initiation of the proliferation assay. No inhibition occurred. It is also possible that HIV antigen, or HIV-induced soluble suppressor factors (12, 19), rather than direct viral infection of T cells accounted for the defect observed. 100 ID_{50} of heat-inactivated (65°C, 1 h) HIV, or cell-free supernatants from 36- and 60-h cultures of infected A57 cells used at a 1:3 (vol/vol) final dilution, were added to fresh A57 lymphocytes plus irradiated HLA-DR2 antigen-presenting cells. No depression of mitogenesis was observed.

Effect of polyI · polyC$_{12}$U on HIV-mediated inhibition of A57 reactivity. Treatment of A57 cells with 250 μg/ml polyI · polyC$_{12}$U before exposure to 100 ID_{50} HIV_B raised the HLA-DR2–specific stimulation index of these lymphocytes to 66% of baseline value in the absence of IL-2 (Table I). In the presence of IL-2, polyI · polyC$_{12}$U raised this index to almost 90% of baseline at both viral loads examined (Table II). In contrast to the minor, DR-2 nonspecific proliferation of HIV-infected cells seen in the absence of polyI · polyC$_{12}$U, no reaction to DR-4/6 was seen in A57 cells infected with HIV in the presence of this molecule.

A57 lymphocytes alone, or in the presence of antigen presenting cells (Table III), were not directly activated by polyI · polyC$_{12}$U.

Effect of HIV on alloproliferation of 86 cells. To evaluate whether HIV-mediated inhibition of alloreactivity is a general phenomenon or is somehow restricted to a particular determinant, the above experiments were repeated using HLA-DR-1 responsive T cells. 1,000 ID_{50} of HIV_B depressed DR-1–driven blastogenesis in 86 cells by a mean of 72% in two experiments

Table II. Effect of HIV on the Alloreactivity of CD4+ Cell Clone A57 Cultured with IL-2 in the Presence and Absence of PolyI · polyC$_{12}$U

Group	HIV	ID_{50}	PolyI · polyC$_{12}$U (250 μg/ml)	Mitogenic response of A57 clone*			Stimulation index‡
				Antigen-presenting cell			
				None	HLA-DR4/6	HLA-DR2	
1	−	—	−	326±86	439±100	6,175±902	18.9
2	+	1,000	−	375±45	1,237±185	687±57	1.83
3	+	100	−	306±33	1,296±58	761±14	2.49
4	+	1,000	+	184±4	222±7	2,997±142	16.3
5	+	100	+	477±59	633±123	7,077±344	14.8

* Conditions as described in Table I, except that cells were cultured for 60 h in the presence of 10% IL-2. ‡ Calculated as described in Table I.

Table III. Effect of PolyI · polyC$_{12}$U on DNA Synthetic Responses of Human Cells to Alloantigen and Mitogen

Cell source*	PolyI · polyC$_{12}$U	Mean [^{3}H]thymidine incorporation‡
	μg/ml	*cpm*
A57	0	15,346±439§
	10	15,502±1,004
	50	14,642±773
	250	12,605±24

		Mean [^{3}H]thymidine incorporation‡		
Cell source*	PolyI · polyC$_{12}$U	Experiment 1	Experiment 2	Experiment 3
	μg/ml	*cpm*	*cpm*	*cpm*
PBMC	0	97,316	20,987	77,170
	10	102,872	23,840	65,387
	50	86,377	23,445	69,481
	250	90,080	27,829	60,698

* Represents cloned CD4+ A57 T lymphocytes cultured in the presence of irradiated HLA-DR2+ antigen-presenting cells, or PBMC obtained from an HIV seronegative donor and cultured with PHA.
‡ Mean counts per minute of triplicate cultures harvested at 36 (A57) or 96 h (PBMC). [^{3}H]Thymidine was included for the last 18 h of incubation.
§ Standard deviation of the mean.

conducted in the absence of IL-2 (Table IV). In the presence of 10% IL-2, alloproliferation decreased by a mean of 64% (Table IV). Similar to the results obtained with clone A57, 250 μg/ml polyI · polyC$_{12}$U raised the stimulation index to > 90% of baseline in the presence of IL-2, and to 74% of baseline in the absence of this lymphokine (Table IV).

In this experiment, as for work with clone A57, cell numbers were calculated based upon viable lymphocytes. To establish with certainty that HIV can directly block alloproliferation in the absence of cytolysis or syncytial cell formation, 86 cell cultures were examined very early after inoculation with virus. 100 ID$_{50}$ of HIV$_B$ was added to 86 cells for 6 h; the cells were washed and either immediately evaluated for proliferation to irradiated DR-1 antigen-presenting cells or incubated for a further 24 or 48 h and examined in a similar manner. These lymphocytes were > 95% viable at all three time points. At 6 h postinoculation, there was no difference in allostimulation between control (6,324±87 cpm of [^{3}H]thymidine incorporation) and infected (5,512±1,003 cpm) cultures. At 24 h, a minor decrease was noted (3,917±515 cpm vs. 2,257±1,003 cpm), whereas at 48 h a profound and sustained depression in alloproliferation occurred (3,919±316 cpm vs. 396±27 cpm).

Inhibition of HIV replication by polyI · polyC$_{12}$U. Repetitive addition of polyI · polyC$_{12}$U to cultures of A57 cells inoculated with 1,000 ID$_{50}$ of HIV$_B$ suppressed the production of mature virions, as detected by assay for particulate reverse transcriptase in culture supernatants. Complete cessation of viral replication occurred at 250 μg/ml polyI · polyC$_{12}$U. To determine if this effect was reproducible in a nonclonal cell population, PHA-activated normal PBMC were exposed to 1,000 ID$_{50}$ of HIV$_B$. Complete inhibition of HIV replication, as defined by lack of incorporation of [^{3}H]thymidine monophosphate into DNA using a poly(rA) · oligo(dT) template, was attained at 250 μg/ml (Fig. 1). The concentration at which the level of infectious virus was decreased by 50% (tissue infectious dose-50) varied from 50–100 μg/ml in three separate experiments.

PolyI · polyC$_{12}$U had no effect when directly added to the assay for reverse transcriptase. It also did not inactivate the polymerase when purified enzyme was incubated with 250 μg/ml polyI · polyC$_{12}$U for 1 h at 4°C before the assay.

Inhibition of HIV-induced cytopathic effects by polyI · polyC$_{12}$U. As an additional check of the antiviral efficacy of polyI · polyC$_{12}$U, its ability to affect HIV-associated cytopathogenicity was examined. 86 cells were maintained in culture alone, together with 250 μg/ml polyI · polyC$_{12}$U, or with this molecule plus 1,000 ID$_{50}$ HIV$_B$ added on culture initiation. Cells were harvested on day 12, and total cell count and viability were assessed by trypan blue dye exclusion. The percent cell recovery was calculated from these data. Cultures not exposed to virus contained 93.2±2.9% viable cells, with polyI · polyC$_{12}$U having no effect on viability or cell recovery (Table V). HIV-infected cultures contained 45.3±11.2% viable cells, whereas those exposed to virus in the presence of polyI · polyC$_{12}$U yielded 77.4±2.6% viable lymphocytes (Table V). Similar results were obtained in parallel experiments with A57 lymphocytes, PHA-activated PBMC, and the CD4+ cell

Table IV. Effect of HIV on the Alloproliferation of CD4+ Cell Clone 86 in the Presence and Absence of PolyI · polyC$_{12}$U

			Mitogenic response of 86 clone‡					
			Without exogenous IL-2			With 10% exogenous IL-2		
			Antigen-presenting cell			Antigen-presenting cell		
Group	HIV*	PolyI · polyC$_{12}$U (250 μg/ml)	None	HLA-DR1	SI§	None	HLA-DR1	SI§
1	−	−	931±68	5,455±202	5.86	1,122±148	6,493±818	5.79
2A	+	−	703±96	979±229	1.39	1,260±120	2,145±576	1.70
2B	+	−	1,079±31	2,094±250	1.94	865±63	2,160±174	2.50
3	+	+	2,063±462	8,904±956	4.32	3,347±156	17,483±212	5.22

* 1,000 ID$_{50}$ of stock HIV$_B$ used to infect cells as described in the legend to Table I. ‡ Values determined as described in Table I. § Stimulation index, calculated as described in Table I.

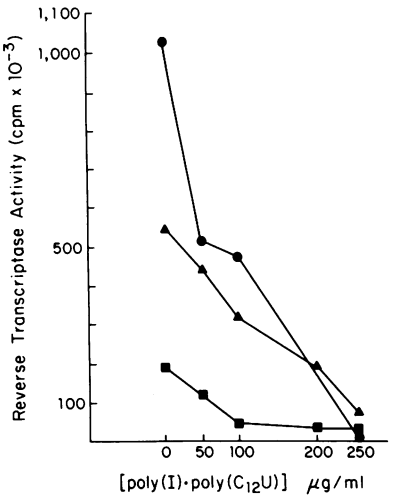

Figure 1. PolyI · $polyC_{12}U$ Inhibition of HIV replication. 1,000 ID_{50} of stock HIV_B were added to 2×10^6 normal PBMC that had been preactivated with PHA for 48 h. Parallel cultures were simultaneously exposed to varying concentrations of polyI · $polyC_{12}U$. Culture medium, including drug, was changed at 48 h and every 3–4 d thereafter. Assays for particulate reverse transcriptase activity were performed on day 6 (■), 13 (▲), and 17 (●). Results are expressed as the mean counts per minute of two determinations of incorporation of [^{3}H]-thymidine monophosphate into acid precipitable material.

line SK. By day 17, infected cultures contained < 5% viable cells, whereas cultures exposed to HIV in the presence of 250 μg/ml polyI · $polyC_{12}U$ were ~ 80% viable.

Another characteristic of HIV infection is the formation of syncytia (13). These multinucleated cells were noted in A57 and 86 cultures within 7 d post HIV exposure, and abrogated in preparations containing polyI · $polyC_{12}U$.

To determine whether continuous exposure of target cells to polyI · $polyC_{12}U$ is required for inhibition of HIV, certain PBMC or A57 cultures were not given additional compound on or after the 6th or 17th d of culture postviral inoculation. Viral activity, defined by reverse transcriptase levels greater than or equal to three times the baseline, was noted on subsequent harvests of culture supernatant taken after the early withdrawal period, but not if polyI · $polyC_{12}U$ had been present through day 17.

HIV trans-activation. The ability of tat_{III} to act at least partly at a posttranscriptional level (20), and the capacity of IFNs and IFN inducers to function at the level of translation in some viral systems, led us to examine the effect of polyI · $polyC_{12}U$ on the activity of this gene. These experiments were initially attempted with A57 and 86 cells and PHA-activated PBMC. Poor cell viability or very low transfection efficiency mandated the use of the CD4+ T lymphoblastoid cell line SK. PolyI · $polyC_{12}U$ had no adverse effect on the enhancement of HIV LTR-promoted CAT gene expression by HIV cDNA clones transfected into SK cells. No conversion of [^{14}C]chloramphenicol into its acetylated forms was noted by transfection of these cells with CAT alone, either in the absence or presence of 250 μg/ml polyI · $polyC_{12}U$. Three acetylated forms of chloramphenicol were detected after co-transfection of SK cells with plasmids containing both the CAT and tat_{III} genes without inhibition in the presence of polyI · $polyC_{12}U$.

As a positive control, a methylphosphonate-modified oligonucleotide complementary to one splice-acceptor site of the bipartite tat_{III} gene was added in place of polyI · $polyC_{12}U$ in a co-transfection assay utilizing the pC15 CAT plasmid and a second vector, pBR322/pIIIextat_{III}, containing the tat_{III} splice-acceptor site (21). This oligomer was capable of blocking tat_{III} enhancement of CAT activity by > 50% (Laurence, J., J. Kulkosky, P. Miller, and P. O. P. Ts'o, unpublished data).

Cellular and HIV-specific proteins. Total cellular and viral proteins were examined in PHA-activated PBMC exposed to HIV. Equal numbers of cells were exposed to HIV in the presence or absence of 250 μg/ml polyI · $polyC_{12}U$. They were metabolically labeled on day 7 and day 10 postinoculation, with equal numbers of counts of radioactivity analyzed by SDS-PAGE. The efficiency of protein precipitation was not affected by the presence of polyI · $polyC_{12}U$ in the reaction mixture. As shown in Fig. 2, there was no obvious difference in individual protein bands in the presence (lanes *3* and *5*) or absence (lanes *2* and *4*) of polyI · $polyC_{12}U$. This was true on both days 7 (lanes *2* and *3*) and 10 (lanes *4* and *5*), periods at which complete suppression of HIV activity had been observed (Fig. 1). Equal numbers of labeled cells were then immunoprecipitated by a human IgG anti-HIV antiserum (17). This reagent is capable of recognizing all major envelope and internal HIV components, with the exception of the 14,000-D tat_{III} protein. As shown in Fig. 3, equivalent amounts of the envelope gp120 and gp160 and group antigen (*gag*) precursor p55 proteins appear to be synthesized in the presence (lane *2*) or absence (lane *1*) of polyI · $polyC_{12}U$. However, the structural protein cleavage product p17 was undetectable and the transmembrane glycoprotein gp41 was markedly reduced in the polyI · $polyC_{12}U$–treated cells.

Table V. Effect of PolyI · $polyC_{12}U$ on HIV-mediated Cytopathology in Clone 86 T Lymphocytes

Group	HIV*	PolyI · $polyC_{12}U$ (250 μg/ml)	Cell viability‡	Viable cell recovery§	Inhibition of viable cell recovery
			%	%	%
1	–	–	93.2±2.9	152.0±9.3	—
2	–	+	92.7±0.7	157.8±32.6	0
3	+	–	45.3±11.2	33.4±5.5	88.0
4	+	+	77.4±2.6	101.1±18.8	35.9

* 0.5×10^6 86 T cells were cultured in the presence or absence of 1,000 ID_{50} of stock HIV_B and polyI · $polyC_{12}U$. Cultures were harvested on day 12. ‡ Mean±standard deviation of two experiments (groups 1, 2, and 4) or four experiments (group 3), measuring percent of cells capable of excluding a 0.2% solution of trypan blue in Hanks'-buffered salt solution plus 1% bovine serum albumin. § Calculated as [(total number of cells) × (percent trypan blue negative cells)]/0.5×10^6. Values represent mean±standard deviation.

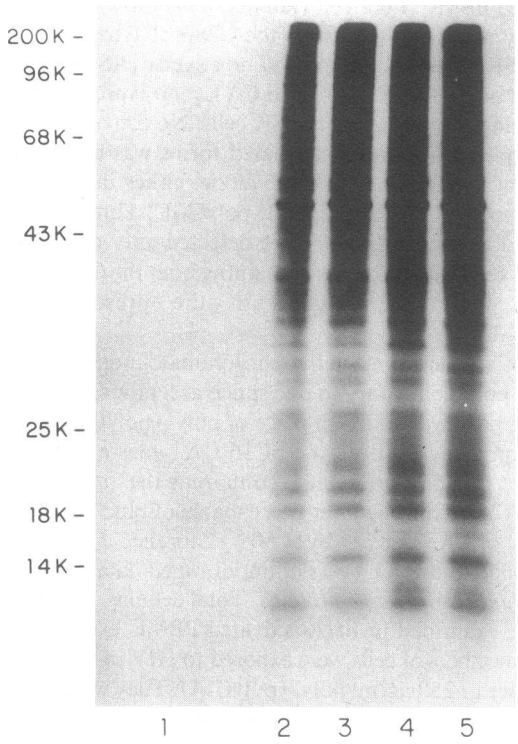

Figure 2. Effect of PolyI · polyC$_{12}$U on proteins synthesized in cells exposed to HIV. PHA-activated PBMC were infected with HIV in the absence (lanes *2* and *4*) or presence (lanes *3* and *5*) of 250 μg/ml polyI · polyC$_{12}$U, as described in Fig. 1. On days 7 (lanes *2* and *3*) and 10 (lanes *4* and *5*) equal numbers of cells were harvested and labeled for 4 h with [^{35}S]cysteine. Equal counts of radioactivity from lysates of these cells were subjected to electrophoresis under reducing conditions on SDS-PAGE and made visible by autoradiography. Lane *1* represents molecular weight markers.

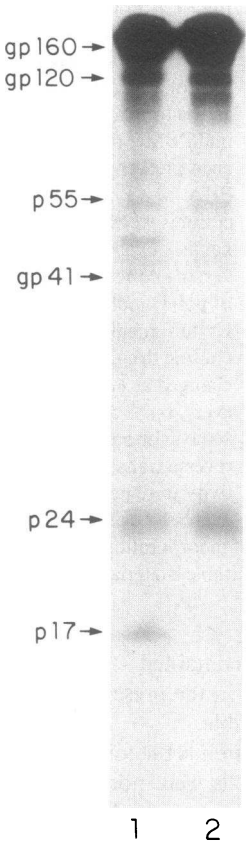

Figure 3. Polypeptide patterns of HIV synthesized in the absence and presence of polyI · polyC$_{12}$U. PHA-activated PBMC were infected with HIV in the absence (lane *1*) or the presence (lane *2*) of 250 μg/ml of polyI · polyC$_{12}$U. On day 7 equal numbers of cells were harvested and labeled for 4 h with [^{35}S]-cysteine. Lysates from these cells were immunoprecipitated with an IgG anti-HIV isolated from the serum of an individual with high titer antibody. They were then analyzed by SDS-PAGE under reducing conditions, as described in Fig. 2.

IFN effects. 1,000 U/ml of anti-human gamma IFN had no effect on HIV inhibition by polyI · polyC$_{12}$U in two separate experiments utilizing PHA-activated PBMC as targets. 1,000 U/ml of anti-human alpha IFN blocked only 21.2% of the inhibitory action of polyI · polyC$_{12}$U on HIV-associated reverse transcriptase (mean of three experiments).

To address the possibility that polyI · polyC$_{12}$U liberated preformed IFNs that were having an antiviral effect in an intracellular milieu protected from interference by anti-IFN antibodies, IFN levels were measured in the PBMC targets. Maximal alpha IFN production was 20 U/ml of culture supernatant, whereas maximal gamma IFN production was 17.3 U/ml.

Discussion

AIDS and ARC patients, as well as asymptomatic HIV carriers, exhibit defective cellular immune responses to antigen, including self and allodeterminants and a variety of viruses, from influenza (22) to HIV (23). In some instances a relative deficiency of MHC self-restricted cytotoxic T lymphocyte responses occurs without loss of cytotoxicity to alloantigen (4, 22). Very low titers of HIV can suppress PBMC blastogenesis to antigen in vitro 2–3 wk postviral exposure (24). These studies all involved unseparated PBMC, representing many cell populations susceptible to HIV infection. We have demonstrated in two clonal CD4+ T lymphocyte lines that loss of alloreactivity is a direct and early consequence of HIV infection. This functional defect was not restricted to one type of class II HLA molecule, as the two clones tested are reactive to distinct HLA-DR specificities.

HIV appeared to alter the function of these clonal T helper cells without destroying the cell. In a preliminary investigation of the mechanism of loss of proliferative capacity in HIV-infected 86 lymphocytes, monoclonal antibodies to T cell surface antigens were used to label these cells in an indirect immunofluorescence assay (12). A > 50% loss of CD4 epitope was noted on day 12. This has been previously reported after infection of CD4+ human lymphoblastoid cells (13) and PBMC (25) with HIV. CD4 antigen expression is not required for helper cell function in certain clones, however (26). Examination of the transduction of membrane signals after antigen exposure in the presence of HIV is being pursued. Monoclonal reagents to the T cell receptor constant region, HLA-DR, transferrin receptor (OKT9), and alpha chain of the IL-2 receptor (tac or CD25) indicate that the respective epitopes were either unaffected or increased after HIV infection. The increase in IL-2 receptor expression, noted in HIV-infected PBMC as well (25), is of particular interest since addition of exogenous IL-2 to our infected T cell clones (Tables II and IV) had no effect on alloproliferation. All of these changes paralleled the appearance of HIV-specific proteins, detected by a

human IgG (17) in an indirect immunofluorescence assay, which was maximal (> 70% membrane-positive 86 cells) on day 12 after HIV exposure.

The inhibition of alloreactivity was unrelated to direct suppression by viral antigen, HIV-induced suppressor lymphokines, or cross-infection of antigen-presenting cells during the blastogenic assay. At very high concentrations (> 10 μg/ml), disrupted HIV virions are inhibitory for T cell proliferative responses to PHA and antigen, resulting in reduced expression of IL-2 receptor (27). However, these levels are not physiologic, with circulating HIV antigen or tissue-associated HIV present at levels of 1–20 ng of protein/ml (28). Heat-inactivated HIV equivalent to immunosuppressive levels of replication-competent HIV (100–1,000 ID_{50}) did not affect either our assays or experiments with PBMC reported by others (24). Also, as we and others have demonstrated (25), infection with intact HIV increases rather than depresses membrane levels of IL-2 receptor. The failure of culture supernatants from HIV-infected A57 and 86 cells to inhibit alloproliferation does not, however, negate the soluble suppressor factor phenomenon described in PBMC from ARC and AIDS patients (12, 19). These lymphokines, elaborated by CD4+ cells, were active only in the presence of viable macrophages (12).

Infection of CD4+ T cell clones with a related retrovirus, HTLV-I, alters their alloreactivity in a different manner, permitting these cells to proliferate nonspecifically to multiple class II determinants (6). This effect was not observed with HIV-infected A57 or 86 cells in the absence of IL-2 (Tables I and IV). In the presence of IL-2, a minor (< 25% of specific stimulation index) response was noted to an irrelevant alloantigen in HIV-infected A57 cells (Table II), but not in infected 86 cells (data not shown).

We used this clonal T cell model to investigate the efficacy and mechanism of a potential antiviral drug, the modified double-stranded RNA polyI · $polyC_{12}U$. Double-stranded RNAs such as polyriboinosinic acid–polyribocytidylic acid (polyI · C) induce IFN and IFN-associated cellular enzymes (29). IFNs inhibit many mammalian retroviruses, including murine leukemia (MuLV), murine sarcoma, feline leukemia, and Mason–Pfizer monkey virus (30). Recombinant alpha-A human IFN (30) and human alpha and beta IFNs (31) can similarly block HIV replication in PBMC and CD4+ T cell lines. PolyI · $polyC_{12}U$, a mismatched analog of polyI · C, is more sensitive to nuclease degradation than the parent molecule and is associated with lower toxicity in clinical trials in animals and man, perhaps because of a more modest production of IFN (29). The effect of polyI · $polyC_{12}U$ is enhanced synergistically by human IFNs in certain model systems (29), indicating that it may exert effects by processes other than IFN induction. This concept was supported by the lack of correlation between IFN and polyI · $polyC_{12}U$ sensitivity in a large number of tumor cell lines screened in vitro and by the inability of anti-IFN antibodies to inhibit the effects of polyI · $polyC_{12}U$ (32).

Our results indicate that polyI · $polyC_{12}U$ is capable of preserving the immune reactivity of clonal CD4+ T lymphocytes exposed to HIV. It apparently does so by inhibition of viral replication. This is demonstrated by the abrogation of HIV-associated cytopathic effects in parallel with loss of reverse transcriptase production and stabilization of alloproliferation. Also, radioimmunoprecipitation and SDS-PAGE analysis of PBMC infected in the presence of polyI · $polyC_{12}U$ indicate that at least certain HIV-specific proteins are synthesized despite the fact that particulate reverse transcriptase is undetectable. The pattern observed is similar to effects seen with IFNs in the MuLV system. Mouse IFN blocks the extracellular appearance of MuLV from chronically infected fibroblasts without altering the level of proviral DNA or viral-specific RNA (33). IFN treatment of lymphocytes chronically infected with Rauscher MuLV leads to changes in the proteolytic cleavage required for proper virion assembly (34, 35). Cleavage of *gag* precursors normally occurs at the time of virion budding or core formation (34). IFN-induced cellular enzymes may disrupt protein glycosylation or phosphorylation, thereby altering the sensitivity of viral peptide precursors to subsequent processing. Alternatively, IFN may have a more general effect on plasma membrane physiology, disturbing the orientation or insertion of viral components in the membrane (34). In the MuLV models there were fewer copies of *gag* cleavage product p30 and more of *gag* p55 than in controls (34). In our experiments with HIV, there appeared to be no *gag* cleavage product p17, substantially less transmembrane cleavage product gp41, and more *gag* p24 in polyI · $polyC_{12}U$–treated cells than in controls (Fig. 3). This occurred despite the fact that equal numbers of cells were used for radioimmunoprecipitation in both treated and control samples, and the levels of envelope components gp160 and gp120 were equivalent in the samples (Fig. 3). Northern blots for HIV-specific messenger RNAs in 86 cell populations infected in the presence of polyI · $polyC_{12}U$ are currently being performed.

IFNs and IFN inducers may also interrupt viral replication at other stages, including translation (36). We have shown that polyI · $polyC_{12}U$ did not affect the function of the tat_{III} gene. The tat_{III} gene product functions only partly at the level of translation (20); transcriptional regulation is also involved (20, 37). Thus, the fact that this molecule did not block HIV-linked *trans*-activation does not preclude a role in interference with translation of viral messages. Since the CAT gene construct we employed was driven solely by an HIV promoter, this experiment does definitively establish that polyI · $polyC_{12}U$ or its induced products do not exert an antiviral effect by involvement with the HIV LTR. This possibility was raised by the recent findings that potential binding sites for regulatory proteins share consensus sequences among gamma IFN, IL-2, HTLV-I (38), and HIV (39, 40).

Whether any of the effects of polyI · $polyC_{12}U$ occurred independently of IFN generation is still uncertain. In our studies, as in other reports (32), antibody to IFNs did not completely block the action of this molecule. Also, the extracellular concentrations of alpha IFN elicited by polyI · $polyC_{12}U$ in our PBMC cultures are insufficient to inhibit HIV replication in PBMC (29, 30). Problems related to production of intracellular IFNs inaccessible to antibody blockade, and the known IFN-inducing potency of the parent compound polyI · C, mandate further investigations.

More than 10 cellular genes have been identified as activatable by IFNs or IFN inducers and are potential mediators of the complex biologic actions of these substances (36). This transcriptional induction is transient even with constant IFN levels, however (36). It may explain our inability to identify new cellular protein synthesis in polyI · $polyC_{12}U$–treated cultures, either at the time points at which we simultaneously

examined HIV-specific peptides (days 7 and 10, Fig. 3) or at 24 h (unpublished data).

A continuous supply of polyI · polyC$_{12}$U appeared essential for complete suppression of HIV activity. This is important therapeutically. It has been suggested that alternating cycles of virus production and IFN synthesis, or administration of IFN or IFN inducers, might be responsible for maintaining a state of chronic retroviral infection (41). With respect to AIDS, another concern is whether a patient's lymphocytes are capable of responding to an IFN inducer. PBMC from individuals with ARC, or AIDS manifest solely by Kaposi's sarcoma, can generate normal levels of alpha IFN in response to herpes simplex virus type I–infected fibroblasts (16).

Our experiments show the utility of CD4+ clonal T lymphocytes with defined antigen reactivity as a model system for the evaluation of HIV-mediated immune suppression as well as anti-HIV agents. The possibility that polyI · polyC$_{12}$U has effects apart from, or in addition to, IFN induction, its synergy with IFNs in other systems, and our studies revealing a parallel between anti-HIV activity and preservation of specific helper T cell function suggest its clinical applicability in HIV infection.

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