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Molecular assessment of the potential combination therapy of cytokines with biphalin and AZT for Friend leukemia virus infection *in vitro*

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Abstract:
Biphalin, a dimeric enkephalin analog, is under investigation as a potential, long-lasting medication of pain associated with chronic diseases, like cancer or AIDS. The role of cytokines, and splenocytes in anti-Friend leukemia virus (FLV) activity of biphalin, a synthetic opioid, and AZT was investigated *in vitro.* Mouse splenocytes inhibited FLV replication in *Mus dunni (Dunni)* cells when they were added to the cell culture. This inhibitory effect of splenocytes also was evident when cells were combined with biphalin and AZT as measured using a focus-forming assay. Under cell-free conditions, recombinant interferon gamma (IFNγ), interleukin 2 (IL-2) and IL-4 directly inhibited the FLV reverse transcriptase (RT) activity by 27% to 36%. IFNγ at 0.005 pg to 500 ng inhibited FLV RT activity by 61% to 80%. A combination of 250 ng IFNγ and 50 μg biphalin resulted in a 94% reduction of FLV RT activity, as compared with 61% inhibition by IFNγ alone. The combination of AZT and IFNγ, IL-2 or IL-4 also induced a stronger suppression of FLV RT activity than either cytokine or AZT used alone. In addition, cloned RT from Moloney murine leukemia virus (MMLV) was directly sensitive to inhibition by biphalin. Thus, the anti-FLV effects of splenocytes in combination with biphalin and AZT in cell culture are likely mediated to a large degree by the direct effect of cytokines. This antiviral activity of splenocytes or cytokines combined with chemotherapy, biphalin, and/or AZT, could be used as a complementary therapy to current approaches for retroviral infection and benefit acquired immunodeficiency syndrome (AIDS) patients. In conclusion, biphalin applied primarily as a new medicine for chronic pain treatment in AIDS patients may play a significant beneficial role as a component of antiviral HIV multidrug therapies.

Key words:
biphalin, 3'-azido-3'-deoxythymidine, cytokine, Friend leukemia virus, opioid, retrovirus

Introduction

Infection with the human immunodeficiency virus 1 (HIV-1) is characterized by a decline in the number of $CD4^+$ lymphocytes and a qualitative impairment of their function [13, 26, 33]. Immunological abnormalities in T helper cell function occur early, during the asymptomatic phase of infection and before the loss in $CD4^+$ cell number [7, 27, 31]. The ability of T cells to recognize and respond to soluble antigen *in vitro* is defective, and they also show spontaneous lymphocyte proliferation, cytokine secretion, expression of activation antigens, and lymph node hyperplasia, suggesting that the T cells are activated [30, 47]. In addi-

tion, the same T cells are susceptible to apoptosis when cultured *in vitro* or upon activation [30]. The loss of T cell function *in vitro* and T cell number *in vivo* predicts progression to acquired immunodeficiency syndrome (AIDS) and a decrease in survival time [11, 35]. 3'-Azido-3-deoxythymidine (AZT), an inhibitor of reverse transcriptase (RT) [32], has been shown to prolong survival in patients with symptomatic HIV infection [14], but it has done little to prevent the progression of AIDS. Currently, highly active antiretroviral therapy with a combination of 2 nucleoside analogue inhibitors plus a protease inhibitor, or nonnucleoside RT inhibitor is undergoing extensive evaluation [39]. While this combination offers improved efficacy, an increase in immune effector cells appears coincident with the reduction in viral load. Analysis of $CD4^+$ cell turnover and lymphoid tissue suggests that redistribution rather than cell proliferation is responsible for the increase [1, 22]. Furthermore, AZT treatments in patients can cause granulocytopenia. Thus, additional therapeutic combinations need investigation.

Interferons (IFN) have been demonstrated to repress HIV replication and syncytia formation in human peripheral blood lymphocytes infected with HIV [49] or HTLV-III replication [18], and IL-2 restored HIV-specific cell-mediated immune responses *in vitro* [6]. Clinical studies also show that IFNγ therapy increases $CD4^+$ cell counts and reduces progression to AIDS as well as opportunistic infections in asymptomatic HIV infected patients [25]. This suggests that AZT therapy is more effective when used with IFNα. Granulocyte-macrophage colony-stimulating factor was also used in combination with AZT for AIDS patients to reduce granulocytopenia caused by AZT treatments in patients [17].

Murine retroviruses have been studied as AIDS models to evaluate the potential efficacy of therapy. Several studies have indicated that AZT could provide some protection against FLV and that it was more effective when combined with immunostimulants [5, 21, 34, 40].

Methionine enkephalin (Met-ENK), an opioid pentapeptide, is known to be an immunostimulator in both mice and humans [48]. The combination of Met-ENK and AZT is more effective than either substance alone in reducing the morbidity and mortality due to the murine retroviruses FLV and BM5 complex [43]. This combination effect was found as an antiviral effect of AZT and an immunostimulatory effect of Met-ENK *in vitro* [41]. In *Dunni* cell culture, an anti-FLV effect of splenocytes treated with Met-ENK in combination with AZT was shown to be mediated, to a large degree, by induction of IFNγ [42]. Thus, stimulation of endogenous Met-ENK expression may play a significant role in antiviral therapy. However, opioid peptide analogs are under development as new opioid analgesics. Particulary biphalin, a dimeric enkephalin analog (Fig. 1) is under development as a new analgesic for chronic, like cancer- and AIDS-related pain. In animal models biphalin expresses high analgesia [20, 23, 28] with low dependence [50] and tolerance [24] development. Biphalin has direct inhibitory effects on FLV replication in cell culture and on FLV RT activity in a cell-free biochemical system, and this effect is enhanced by the combination of biphalin with AZT [45].

Here, we show that splenocytes and some cytokines are directly involved in inhibiting FLV focus formation in cell culture and viral RT activity in a cell-free biochemical assay alone or in combination with biphalin and AZT. Combination of mouse splenocytes or cytokine with biphalin or AZT resulted in increased antiviral inhibitory effects as compared with either drug used alone. Thus, it appears that cytokines act in concert with AZT and/or biphalin, as antiretroviral therapy, *via* the inhibition of viral RT activity.

Materials and Methods

Materials

Recombinant mouse IFNγ, IL-2 and IL-4 were purchased from Genzyme (Cambridge, MA). These were aliquoted in PBS and then stored at 4°C until used. Biphalin, a dimeric enkephalin analog (Fig. 1) has been synthesized by one of us (A.W.L.) by the method described previously [28]. It was suspended in PBS solution for use in tissue culture and cell-free testing. 3'-Azido-3'-deoxythymidine (AZT) was supplied in powder form by Burroughs Wellcome Co. (Glaxo, Research Triangle Park, NC). Cloned MMLV RT was purchased from Amersham Life Science (Cleveland, OH).

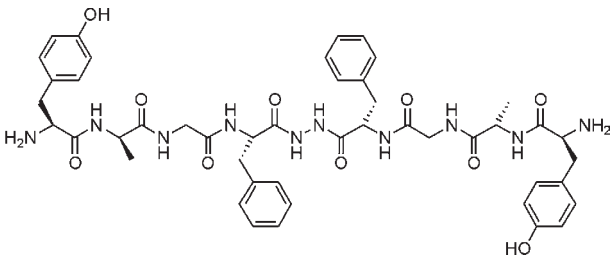

Fig. 1. Molecular formula of biphalin

Virus

Friend leukuemia virus (FLV) was the N/B tropic polycythemia inducing strain that contains the entire FLV complex, composed of a helper lymphocytic leukemia virus and a defective spleen focus forming virus. Virus was propagated by infecting BALB/c mice and harvesting spleens 21 days post-infection. Samples were homogenized in Hanks balance salt solution (HBSS; Sigma, St. Louis, MO), quantified using a focus forming assay and stored at –70°C until use.

Cell line

Dunni cells, kindly provided by Dr. Bruce Chesebro (NIH Rocky Mountain Labs, Hamilton, MT), are fibroblasts obtained from the wild mouse, *Mus dunni.* The cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS, Sigma), 100 units penicillin and 100 μg streptomycin per ml and 0.25% 1 M HEPES buffer solution (Mediatech, Washington, DC) in 25 cm^2 plastic tissue culture flasks (Costar, Cambridge, MA) at 37°C in 5% CO_2, 95% air. For testing drug effects, the cells were incubated in 24-well cluster plastic plates (Falcon, Lincoln Park, NJ) at 1×10^5 cells/well in 0.5 ml RPMI 1640 with 5% FBS for 1 day, then the medium in the culture was diluted by adding 0.5 ml FBS-free medium for an additional period of 3 days. Cell cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2, 95% air.

Splenocyte collection

Spleens were aseptically removed from BALB/c mice, and a single cell suspension was made as previously described [42]. For the focus forming assay, 10^6 splenocytes were added to each well after *Dunni* cells were infected with FLV for 2 h.

Focus forming assay

Virus replication was detected by measuring focus forming units (FFU) on *Dunni* cells. Cells in 24-well plates were infected with FLV (approximately 50–100 FFU) for 3 days, the medium in the culture was removed then, 0.2 ml of monoclonal anti-FLV gp70 antibody [4] was added per well, the cell cultures were incubated at 4°C for 30 min. Cells were then washed twice with PBS containing 1% FBS and fixed with methanol (Sigma) for 5 min at room temperature. Cultures were rinsed twice with PBS and 0.2 ml of goat anti-mouse IgG labeled with horseradish peroxidase was added into each well. The cell cultures were further incubated at room temperature for 40 min and rinsed twice with TNE (0.01 M Tris HCl, 0.15 M NaCl, 0.002 M EDTA, pH 7.5). Stock solution of 3-amino-9-ethyl-carbazole (Sigma) in dimethylformamide (4 mg/ml) was freshly diluted 1: 19 with 0.05 M sodium acetate-acetic acid buffer, pH 5, and then added into the cultures immediately. After 20 min of incubation at room temperature in the dark the cultures were rinsed and the amount of FLV was determined by counting the number of foci in each well.

Reverse transcriptase (RT) assay

RT activity was measured as previously described [33]. Briefly, the RT assay mixture contained 5 μl of Tris (1 M, pH 8.0), 2.5 μl of $MnCl_2$ (0.04 M), 10 μl of KCl (1 M), 5 μl dithiothreitol (0.1 M), 1 μl of primer-template [1 mg of poly(rA) $p(dT)_{12–18}$ (Pharmacia, Piscataway, NJ) per ml], and 1 μl of ^{3}H-TTP (1 mCi of [methyl-^{3}H]TTP [Amersham, Arlington Heights, IL] per ml) as well as FLV or cloned MMLV RT and DEPC-treated H_2O with or without biphalin and/or AZT. Reaction mixtures were incubated at 37°C for 30 min. Polymerized ^{3}H-TTP was precipitated with 10% trichloroacetic acid and was separated from free ^{3}H-TTP by centrifugation (14,000 rpm, 4°C, 5 min). The pellets of polymer with ^{3}H-TTP were dissolved in DEPC-treated water and radioactivity was assayed using a liquid scintillation spectrometer.

Results

Mouse splenocyte effect

Because immune cells play an important role in HIV infection, mouse splenocytes were tested for their direct effect on FLV replication in cell culture. When 10^6 splenocytes were added to *Dunni* cell cultures that were infected 2 h earlier with FLV, viral replication was decreased by 36% (Tab. 1). Since biphalin has been shown previously to have an inhibitory effect on FLV, splenocytes were combined with biphalin at the same concentration as either used alone. The suppression of FLV replication using the combination was 58%, which was greater than either biphalin or splenocytes used alone.

Tab. 1. Inhibitory effect of mouse splenocytes and biphalin on the replication of Friend leukemia virus

Treatment	FFU (± SD)	Inhibition (%)
None	91 + 1	–
Biphalin (100 μg/ml)	54 ± 6*	41
Splenocytes (1 × 10^6/ml)	58 ± 5*	36
Biphalin & Splenocytes	38 ± 1**	58

Cells were cultured in 24-well cluster plates at 5 × 10^4 cells per well in RPMI 1640 with 5% FBS for 1 day. Cultures then were supplemented with FBS-free medium and polybrene (8 μg/ml), and virus. After 2 h of incubation, biphalin and/or splenocytes were added into the cultures. Three days later, the virus in the cells was quantified using the focus forming assay and expressed as the mean ± SD in focus forming unit (FFU). Inhibition (%) was calculated as: [(FFU without treatment – FFU with treatment)/FFU without treatment] × 100. Three replicates were used for each group. * Statistically significant at $p < 0.05$ using Student's *t*-test compared with control. ** Statistically significant at $p < 0.05$ using Student's *t*-test compared with either biphalin or splenocytes treatments alone. Representative data from one of 3 experiments

Effect of mouse splenocytes combined with biphalin and AZT

Mouse splenocytes were also tested in combination with biphalin and AZT in the FLV focus formation assay using *Dunni* cell cultures (Tab. l, 2). One million splenocytes were added with or without biphalin and AZT to *Dunni* cell cultures that had been infected 2 h earlier with FLV. Biphalin and AZT treatment inhibited viral replication by 47% when compared with untreated cultures, which was a significant reduction compared with AZT alone (38%) or biphalin alone (22%). Addition of splenocytes combined with biphalin and AZT into cell culture resulted in the greatest inhibition in the focus forming assay (68%).

Tab. 2. Inhibitory effect of biphalin combined with splenocytes and/or AZT on the replication of Friend leukemia virus

Treatment	FFU (± SD)	Inhibition (%)
None	117 ± 12	–
Biphalin (100 pg/ml)	91 ± 9*	22
AZT (1 ng/ml)	73 ± 9*	38
Splenocytes (1 × 10^6/ml)	51 ± 7*	57
Biphalin & AZT	62 ± 6**	47
Biphalin & AZT & Splenocytes	37 ± 6***	68

The experimental protocol was the same as for Table 1. Inhibition (%) was calculated as: [(FFU without treatment – FFU with treatment)/FFU without treatment] × 100. Three replicates were used for each group. * Statistically significant at $p < 0.05$ using Student's *t*-test compared with control. ** Statistically significant at $p < 0.05$ using Student's *t*-test compared with either biphalin or AZT alone. *** Statistically significant at $p < 0.05$ using Student's *t*-test compared with both splenocytes alone and biphalin plus AZT treatment combined. Representative data from one of 3 experiments

Cytokines effect on FLV RT activity

The ability of cytokines to directly reduce FLV RT activity was tested in a cell-free system. As a negative control, cytokines did not stimulate much RT activity (CPM) in precipitates, when they were present in the RT mixture solution without FLV (Tab. 3). After these counts were subtracted from the counts of the RT mixture in the presence of cytokines in solution with FLV, IL-2, IL-4 or IFNγ reduced FLV RT activity by 27% to 36%, which was significant when compared with RT activity demonstrated by FLV alone. Although the reduction of RT activity by IFNγ was slightly greater than IL-2 or IL-4 at the same concentration, a statistically significant difference was not noted using the Student's *t*-test.

IFNγ effect on FLV RT activity

The effects of different concentrations of recombinant IFNγ on FLV RT activity are presented in Table 4. In the absence of virus, 0.005 pg to 500 ng of IFNγ alone, caused no alteration of background activity. A decrease in RT activity was noted when IFNγ was

added into the mixture solution containing FLV, but there was no dose-related effect. Table 4 also shows the capacity of biphalin combined with IFNγ to inhibit the FLV RT activity. Biphalin (50 μg) combined with 250 ng of IFNγ resulted in a stronger inhibitory effect, than 500 ng of IFNγ added alone, decreasing RT activity by 94% overall.

Tab. 3. Inhibitory effect of cytokines on the RT activity of Friend leukemia virus

Virus	Cytokine	Count (CPM ± SD)	Specific counts (CPM)	Inhibition (%)
None	none	445 ± 60	–	–
FLV	none	2,874 ± 106	2,429[&]	–
None	IL-2	944 ± 19	–	–
None	IL-4	994 ± 5	–	–
None	IFNγ	745 ± 55	–	–
FLV &	IL-2	2,707 ± 64*	1,763	27[H]
FLV &	IL-4	2,623 ± 85*	1,629	33
FLV &	IFNγ	2,310 ± 92*	1,565	36

The RT mixture contained 500 FFU of FLV and 50 ng of IL-4 or IFNγ, or 100 ng of IL-2. Total volume of each pellet of samples was divided into three counting tubes containing 3 ml of scintillating solution for measurement of the radioactivity. The data are expressed as the mean ± SD of these three tubes. [&] Specific counts present RT activity of FLV and were calculated as: counts of FLV CPM – background CPM, or counts of FLV & cytokine CPM – counts of cytokine alone CPM. [H] Inhibition is expressed as a percentage compared to specific counts for FLV alone: [(FLV CPM – FLV & cytokine CPM)/FLV CPM] × 100. * Statistically significant at $p < 0.05$ using Student's *t*-test compared with FLV alone. Representative data from one of 3 experiments

Effect of cytokines combined with AZT on FLV RT activity

The effect of cytokines combined with AZT on FLV RT activity was further examined in the cell-free RT system. FLV RT activity was inhibited by AZT as shown in Table 5. The diminution of RT activity was enhanced when AZT was combined with each of the cytokines tested. Again there was no significant dose effect for any of the cytokines tested.

Biphalin effect on cloned MMLV RT activity

In parallel experiments, cloned RT of MMLV was used to examine directly the effect of biphalin on purified viral RT, whereas the FLV experiments assessed the RT activity extracted from the whole virus (Tab. 6). Biphalin (500 ng) alone, in the solution without MMLV RT did not apparently alter background RT activity, compared with the negative control. However, when 500 ng of biphalin were added to the MMLV RT mixture solution, activity was reduced by 34% compared with MMLV RT alone.

Tab. 4. Inhibitory effect of interferon gamma with or without biphalin on the RT activity of Friend leukemia virus

Virus	IFNγ	Biphalin	Counts (CPM ± SD)	Specific counts (CPM)	Inhibition (%)
None	none	none	1,197 ± 32	–	–
FLV	none	none	3,058 ± 52	1,861[&]	–
None	0.005 pg	none	1,608 ± 106	–	–
None	500 pg	none	1,683 ± 127	–	–
None	500 ng	none	1,627 ± 24	–	–
FLV &	0.005 pg	none	1,973 ± 86*	365	80[#]
FLV &	500 pg	none	2,033 ± 113*	350	81
FLV &	500 ng	none	2,355 ± 174*	728	61
FLV &	250 ng &	50 μg	1,738 ± 148*,**	111	94 85[I]

The experimental protocol was the same as for Table 3 except that RT mixture contained the indicated concentrations of reagents and 50 FFU of FLV. [&] Specific counts present RT activity of FLV and were calculated as: counts of FLV CPM – background CPM, or counts of FLV & reagent(s) CPM – counts of IFN alone CPM. [#] Inhibition is expressed as a percentage compared to specific counts for FLV or IFN alone [(FLV CPM – FLV & reagent(s) CPM)/FLV CPM] × 100. [I] Inhibition is expressed as a percentage compared to specific counts for FLV & INF: [(FLV & IFN 500 ng CPM – FLV & IFN & biphalin CPM/FLV & IFN 500 ng CPM] × 100. * Statistically significant at $p < 0.05$ using Student's *t*-test compared with FLV alone. ** Statistically significant at $p < 0.05$ using Student's *t*-test compared with FLV & IFN 500 ng. Representative data from one of 3 experiments

Tab. 5. Inhibitory effect of cytokines and AZT on the RT activity of Friend leukemia virus

Virus	AZT	Cytokine	Counts (CPM ± SD)	Specific counts (CPM)	Inhibition (%)	
None	none	none	749 ± 54	–	–	
FLV	none	none	3,421 ± 247	2,672[&]	–	
None	AZT	none	574 ± 38	–	–	
None	AZT &	IFNγ (5 ng)	641 ± 29	–	–	
FLV &	AZT	none	3,074 ± 12*	2,500	6[#]	
FLV &	AZT &	IFNγ (50 pg)	2,448 ± 102*,**	1,807	32	27[I]
FLV &	AZT &	IFNγ (5 ng)	2,612 ± 160*,**	1,971	26	21
FLV &	AZT &	IL-2 (50 pg)	2,502 ± 30*,**	1,861	30	26
FLV &	AZT &	IL-2 (5 ng)	2,571 ± 34*,**	1,930	28	23
FLV &	AZT &	IL-4 (0.1 ng)	2,397 ± 190*,**	1,756	34	30

The experimental protocol was the same as for Table 3 except that RT mixture contained the indicated concentrations of reagents and 50 FFU of FLV. [&] Specific counts present RT activity of FLV and were calculated as: counts of FLV CPM – background CPM, or counts of FLV & reagent(s) CPM – counts of AZT or AZT & IFN CPM. [#] Inhibition is expressed as a percentage compared to specific counts for FLV alone as: [(FLV CPM – FLV & AZT CPM)/FLV CPM] × 100. [I] Inhibition (%) is expressed as a percentage compared to specific counts for FLV & AZT as: FLV & AZT CPM – FLV & AZT & cytokine CPM)/FLV & AZT CPM] × 100. * Statistically significant at $p < 0.05$ using Student's *t*-test compared with FLV alone. ** Statistically significant at $p < 0.05$ using Student's *t*-test compared with FLV & AZT. Representative data from one of 3 experiments

Tab. 6. Inhibitory effect of biphalin on the activity of cloned RT of Moloney-murine leukemia virus

RT	Biphalin	Counts (CPM ± SD)	Specific counts (CPM)	Inhibition (%)
None	none	2,040 ± 74	–	–
MMLV	none	9,533 ± 1,372	7,493[&]	–
None	500 ng	1,316 ± 189	–	–
MMLV &	500 ng	6,235 ± 243*	4,919	34[#]

The experimental protocol was the same as for Table 3 except that RT mixture contained the indicated concentrations of biphalin and/or 960 ng of cloned RT of MMLV. [&] Specific counts present the activity of cloned RT of MMLV and were calculated as: counts of cloned RT CPM – background CPM, or counts of cloned RT & Biphalin CPM – counts of Biphalin CPM. [#] Inhibition is expressed as a percentage compared to specific counts for cloned RT alone as: [(cloned RT CPM – cloned RT & Biphalin CPM)/cloned RT CPM] × 100. * Statistically significant at $p < 0.05$ using Student's *t*-test compared with cloned RT alone. Representative data from one of 3 experiments

Discussion

AIDS is a secondary immune deficiency caused by infection with HIV. Virus-specific $CD4^+$ T helper lymphocytes are critical to the maintenance of effective immunity in a number of chronic viral infections, but are characteristically greatly decreased in chronic HIV-1 infection. HIV infects and kills $CD4^+$ helper T cells as well as dendritic macrophages, resulting in a profound, irreversible immunosuppression that progresses to death due to fatal opportunistic infections in most infected individuals [38, 44]. $CD8^+$ lymphocytes have been shown to inhibit replication of HIV *in vitro* when co-cultured with HIV-infected $CD4^+$ lymphocytes. This suppressive effect on HIV replication in experimentally infected CD4 cells has so far been demonstrated not only for CD8 cells from HIV-seropositive individuals but also for CD8 cells from HIV-seronegative individuals. There are no differences between CD8 cells from HIV-positive and -negative individuals in their suppressive capacity for HIV replication [37]. In our experiments, we document that splenocytes from mice not exposed to FLV can suppress virus replication in *Dunni* cells (Tab. 1 and 2), supporting that the mechanism(s) involved is/are not virus-induced specific immune responses and may be due to vigorous non-specific cytokine responses. Previous studies in our laboratory using *Dunni* cells in an FLV infection system *in vitro* have demonstrated that in the presence of Met-ENK, splenocytes were stimulated to release IFNγ to inhibit FLV replication [41]. This suggested that another opioid peptide with active molecular residues similar to Met-ENK, biphalin combined with splenocytes and/or AZT might be involved in stimulating immune cells to suppress FLV replication in cell culture. The putative factor(s) re-

sponsible for the suppressive mechanism may involve cytokines. Additional studies need to be performed to determine whether biphalin induces the release of cytokines to contribute to this effect. The increased inhibitory effect noted when biphalin was combined with splenocytes and AZT extends our earlier findings that biphalin and AZT have antiviral activity. The experimental design used in this study also demonstrated the direct suppression of FLV RT activity by cytokines, AZT and/or biphalin (Tab. 3 and 4) that also extend a previous report [45] showing that cytokines and biphalin inhibit FLV RT activity.

CD4 helper T (Th) cells are characterized by the patterns of cytokines they produce after activation. IL-4 is produced by Th2 cells, but Th1 cells produce IL-2 and IFNγs which are responsible for cell-mediated immunity against intracellular infection [2]. Thus, cytokines may also be thought to be a part of intrinsic immune properties of T cells. HIV infection may be related to the change in these cells' intrinsic immune properties. Cloned $CD4^+$ T cells from AIDS patients have a reduced ability to produce IL-2 and IFNγ in response to activation with phytohemagglutinin [29]. Furthermore, the expressions of Th1 and Th2 cytokines are changed in mice with murine AIDS [16]. Clinically, a subset of HIV-1-infected individuals who appear to successfully control virus replication in the absence of antiretroviral therapy has been identified [36]. In these individuals, the exposure of lymphocytes to HIV-1 antigen resulted in the specific induction of IFNγ production. It seems likely that sufficient or vigorous cytokine production by immune cells is important in the immune response needed to inhibit virus replication. Since the location of cytokines in the cell cytoplasm is also considered as the place of viral RT induced transcription, a direct interaction between cytokines and viral RT activity was examined using a cell-free RT assay. Direct inhibition of FLV RT activity by IL-2, IL-4 and IFNγ (Tab. 3 and 4) suggests that levels of these cytokines in immune cells may participate directly in controlling retrovirus replication.

Since the introduction of highly active antiretroviral therapy (HAART), there has been a decrease in the incidence of opportunistic infections among HIV infected patients along with a corresponding reduction in the mortality rate [9, 15]. However beneficial HAART has been, experience during the past several years has disclosed the emergence, in a small proportion of cases, of a unique set of complications. The frequency of productive mononuclear cells (MNCs) initially diminished but was still detectable, as was proviral DNA [3]. Indeed, many fundamental questions about the new anti-HIV drugs are still unresolved [8, 10, 39].

Increased inhibition of FLV RT activity by IL-2, IL-4 and IFNγ in combination with AZT and/or biphalin provides further stimulus for the need to correlate intracellular molecular interaction between FLV infection and therapeutic combination (Tab. 5). The data presented here extend the understanding of and need for using the cell-free viral RT inhibition reaction as an *in vitro* assay of the combination therapy to retroviral infection. Emphasis is placed on noting that the cell-free assay is a measure only of the drug-virus interaction between FLV RT and therapeutic agents. Other factors such as dose, strain and route of infection, the stability of the infectivity in the host, and the efficiency of drug delivery to the infected immune systems and into the cells are all likely to be important *in vivo*. Nonetheless, some degree of drug-virus interaction is likely to be essential in HIV infection and progression to AIDS, especially given the remarkable correlation between virus replication (viral RT activity) and T cell dysfunction (Tab. 3 and 4). These latest data may have important implications for HIV therapy and pathogenesis. Since rapid turnover of HIV-1, the decrease or insufficiency of the intrinsic immune inhibition of virus replication, generation of viral diversity and the attendant increased opportunities for viral escape from therapeutic agents are unavoidable sequelae, therapeutic strategies must, therefore, be initiated using optimal combination(s) of agents to help rescue or rebuild the intrinsic immune properties of T cells.

Interventional approaches to limit AIDS pathogenesis have shown that HIV-1 production and clearance are delicately balanced but involve highly dynamic processes [19, 46]. Our results show that these dynamic processes may be affected by many factors including direct interaction between viral RT activation and suppressive molecules (Tab. 3–6). Taken together, these findings strongly support the view that cytokines and antiviral drugs used in the proper combination can be high-level inhibitors of FLV replication as well as immune stimulants leading to therapeutic benefits in FLV infection and perhaps may be extended to HIV in humans.

In addition, HIV/AIDS-related pain remains a clinically challenging condition despite recent advances in treatment modalities. The existing data on pain in HIV-positive persons demonstrate a high prevalence,

wide variability in clinical presentation, significant negative impact on health-related quality of life, and alarmingly inadequate assessment and management [12]. Opioid peptide analogs have been proposed as a new generation of medicines for chronic pain treatment. The presented data evidence that application of new analgesic medicines, like biphalin could be beneficial not only in respect of prolonged effective analgesia but also as a significant component of multidrug therapy of HIV infections.

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