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DIFFERENTIAL MODULATION OF HOST CELL AND HIV GENE EXPRESSION BY COMBINATIONS OF AVAROL AND AZT *IN VITRO*

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Abstract—Human immunodeficiency virus type 1 (HIV-1) gene expression is modulated by some virus-encoded proteins, possibly acting at multiple levels of control, which are also known to be involved in the regulation of gene expression in uninfected cells (transcriptional, post-transcriptional, nucleocytoplasmic transport, and translational control). Two anti-HIV-1 drugs, Avarol and 3′-azido-3′-deoxythymidine, which inhibit viral replication by differential mechanisms, were used to study the role of cytoplasmic factors in independent regulation of host cell and viral gene expression. Both drugs were found to inhibit viral replication and synthesis of virus-encoded protein in a synergistic manner, while at cytostatic concentrations, both compounds act antagonistically. ATP-induced transport of viral messengers from isolated nuclei is enhanced by total cytosolic protein from HIV-1-infected cells; a strong increase of the nucleocytoplasmic transport of *pol* mRNA was measured and, to a lesser extent the transport of certain cellular mRNA (e.g. interleukin-2) was augmented, while the transport of other cellular mRNA (actin) was not affected at all.

It is now well established that human immunodeficiency virus (HIV) is the etiological agent of the acquired immune deficiency syndrome (AIDS) [1, 2]. The genomes of the human T-lymphotropic (or leukemia) viruses HTLV-I and II, HIV-1 (HTLV-III) and HIV-2 contain in addition to the viral replication genes *gag*, *pol* and *env* extra genes, which are not homologous to mammalian genes (e.g. *onc* genes) [3]. Recent results show that HIV-1 contains at least five extra genes [4]; the corresponding gene products are assumed to be involved in the regulation of viral gene expression at multiple levels (transcription, mRNA processing, translation and virus maturation) [5, 6]. Based on transfection experiments it has been proposed that the gene product of *tat*-III (*trans*-activator gene) enhances expression of genes linked to the HIV-1 long terminal repeat (LTR), while the gene product of 3′-*orf* (3′ open reading frame) is the corresponding negative regular [7, 8]; a further regulatory gene product is present, *trs* (*trans*-acting regulator of splicing), which has the proposed function to regulate the relative steady-state levels of HIV-1 genomic and spliced subgenomic mRNA [5]. The molecular events through which these regulatory gene products control viral gene expression are unknown. An elucidation of their mechanisms of action is required (i) to understand the flexibility and plasticity of adaptive regulation of viral gene expression in the human host cells and (ii) to permit a rational development of novel chemotherapeutic agents. In this contribution we used the two anti-HIV drugs, Avarol [9] and 3′-azido-3′-deoxythymidine (AZT) [10], to demonstrate that these compounds differentially interfere with the mechanisms involved in HIV versus cellular gene expression. On the other hand, we show that cytoplasmic viral gene products differentially act on nucleo-cytoplasmic efflux of viral and cellular mRNA.

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MATERIALS AND METHODS

The human serum from an AIDS patient containing antibodies directed against the gp160, gp120, p66, p55, p51, gp41, p31, p24, p17 and p14 virus encoded proteins (p) or glycoproteins (gp) was a gift of Prof. K. H. Meyer zum Büschenfelde and Prof. G. Hess (I. Medizinische Klinik, Universität Mainz).

Cell culture and infection with HIV-1. The details of cultivation of CD4 (T4/Leu3) positive cell line H9 and infection with the HIV-1 strain HTLV-IIIB were given earlier [9, 11]. H9 cells (4×10^5 cells/ml) were infected with 2×10^8 HTLV-IIIB particles and routinely cultivated for 4 days. The total number of viable cells per assay was determined electronically (Cytocomp Counter; model Michaelis); the percentage of cells expressing p24 *gag* protein of HIV-1 was determined by indirect immunofluorescence microscopy [9]; as a measure of viral titers the activity of reverse transcriptase was determined in the supernatants [11, 12]. The number of doubling steps was calculated as described [13].

The 50% effective doses (ED_{50}) which inhibit virus growth by 50% as well as the 50% inhibitory doses (ID_{50}) displaying a 50% antiproliferative activity were

determined as described [14]. The ED_{50} and the ID_{50} concentrations for selected combinations of AZT and Avarol were plotted according to the isobologram procedure [15, 16]. The fractional inhibitory concentration index (FIC) was determined as described [16, 17].

Messenger RNA efflux measurements. Nuclei were isolated from the cells by the method described by Blobel and Potter [18], except that 5 mM 2-mercaptoethanol and 1 mM phenylmethanesulfonyl fluoride were added to all the buffers used. Incubation of the isolated nuclei for 30 min at 30° in transport medium (25 mM Tris–HCl, pH 7.6; 250 mM D-glucose, 2 mM $MgCl_2$, 0.5 mM $CaCl_2$, 0.3 mM $MnCl_2$, 5 mM spermidine, 5 mM 2-mercaptoethanol, and 10^3 units/ml of RNasin) supplemented with 2 mM ATP plus an ATP regenerating system (35 units/ml of pyruvate kinase, 5 mM phosphoenolpyruvate, and 5 mM Na_2HPO_4) was performed as described previously [19, 20], in the absence and presence of total cytosolic protein from HTLV-IIIB-infected H9 cells (100,000 *g* supernatant of an H9 cell homogenate, dialyzed against 50 mM Tris–HCl pH 7.6, 2.5 mM $MgCl_2$, 25 mM KCl). The cellular and viral mRNA in the total postnuclear supernatant from 4×10^5 nuclei was analyzed by dot-blot hybridization.

RNA isolation and blot hybridization. Total RNA was isolated [21] and 2.5 μg of RNA each were applied to a nitrocellulose sheet (BA85; Schleicher & Schuell) [22] and hybridized with one of the following ^{32}P-labeled probes: β-actin DNA, IL-2 DNA, IFN-γ DNA or HTLV-IIIB *pol* DNA. The human β-actin gene [600-bp 3′UTP fragment [23]] and the human pre IL-2 (T-cell growth factor) cDNA [24] were cloned into pBR322, and the human IFN-γ cDNA [25] and the *pol* fragment [nucleotide position 1257 (*Hin*dIII restriction site) to position 4238 (*Eco*RI site), according to nucleotide sequence of HTLV-III published by Ratner *et al.* [26]] were cloned into pUC vector. They were labeled with [α-^{32}P]dTTP by nick-translation [27] to a specific activity of

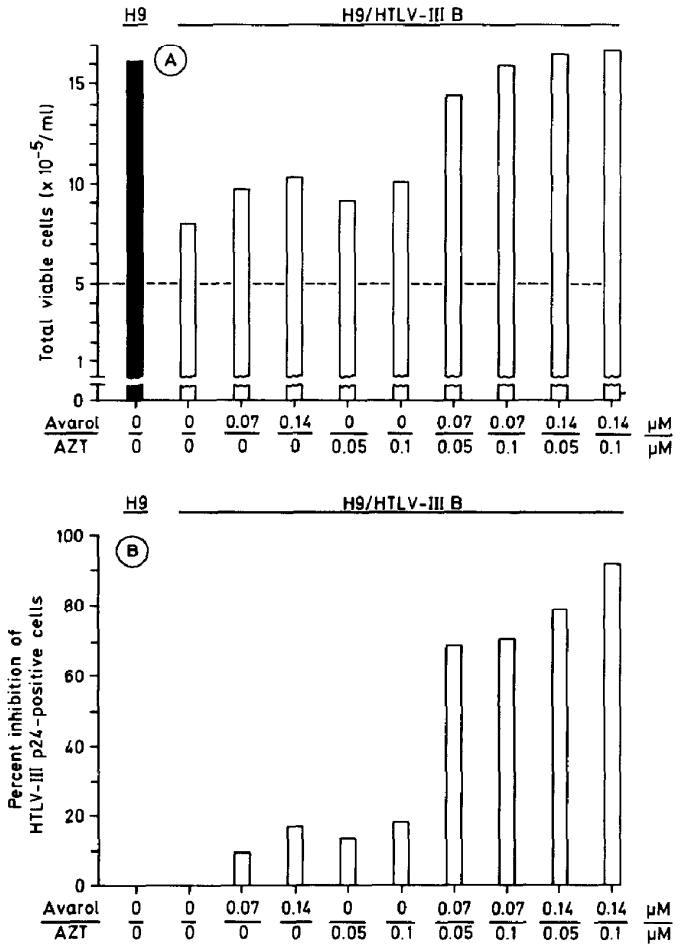

Fig. 1. Inhibition of cytopathic effect (A) and inhibition of HIV-1 expression (B) by Avarol or AZT (either given alone or in combination) *in vitro*. H9 cells were infected with HTLV-IIIB particles and cultivated for 4 days in the absence or presence of compound. Then the total viable cells per assay (A) and the percent inhibition of cells expressing p24 *gag* protein of HIV-1 were determined (B); the percentage of HIV-1 p24-positive cells was determined by indirect immunofluorescence microscopy with the use of a mouse monoclonal antibody to p24. The dotted horizontal line in A shows the starting number of uninfected or virus-infected H9 cells. All bars represent the mean value of duplicate assays.

7×10^7 cpm/μg DNA. The dried filters were exposed to Kodak XAR-5 X-ray film.

Dot-blot hybridization was performed as described [22].

RESULTS AND DISCUSSION

It has been well established that AZT [28], converted into its triphosphate form, inhibits preferentially HIV reverse transcriptase, very likely by acting as a chain terminator for DNA synthesis [10, 29]. Avarol [30], which does not affect DNA- or RNA polymerases, abolishes HIV replication by an interference with the expression of the viral protease gene [31]. Experiments were performed with the cell clone H9, an OKT4$^+$ T-cell line that is permissive to HTLV-IIIB replication [9, 11]. As summarized in Fig. 1, both Avarol and AZT display a significant anti-HIV effect at concentrations as low as 0.07–0.14 μM for Avarol or 0.05–0.10 μM for AZT, as judged on the basis of inhibition of (i) cytopathic effect of HTLV-IIIB on H9 cells (Fig. 1A) and (ii) HIV-1 p24 *gag* protein expression (Fig. 1B). As an example, in the presence of 0.14 μM of Avarol or 0.10 μM of AZT HTLV-IIIB-infected H9 cells performed 1.01 and 1.03, resp. doubling steps after a 4-day exposure to the drugs; in the absence of the drugs, the number of doubling steps was only 0.64. However, after coaddition of these two compounds, the proliferation rate was 1.75, a value which is characteristic for uninfected H9 cells (1.70) (Fig. 1A). Thus the cytoprotective effect of the two compounds, given in combination, was synergistic. The same effect was observed if the inhibition of HIV-1 p24 *gag* expression in H9 cells was chosen as a parameter (Fig. 1B). A quantitative assessment of the combined effects of Avarol and AZT on HIV replication was achieved by using the viral reverse transcriptase activity in the culture supernatants of HTLV IIIB-infected H9 cells as an indicator (Fig. 2B). The results revealed that after a 4-day incubation period, 0.21 ± 0.02 μM of Avarol or 0.13 ± 0.02 μM of AZT reduced the enzyme activity to 50% (ED_{50}), reflecting a 50% inhibition of HIV replication. Dose–response curves for Avarol and AZT were obtained to determine the concentration required for 50% inhibition of HIV replication when tested in combination. The amounts of each drug required were plotted on an isobologram (Fig. 2B). The results revealed a pronounced synergism between the two drugs, indicated by the curve obtained to the left of an imaginary straight line joining the ED_{50} concentration value for each drug alone, e.g. the fractional inhibitory concentration index (FIC) at the drug combination 0.025 μM of AZT and 0.033 μM of Avarol revealed a value of 0.35, indicating significant synergism ([32]; additive effects: FIC = 1; antagonism: FIC > 1).

In constrast to the synergistic effect determined for Avarol and AZT against HIV replication in H9 cells, the two compounds affect growth of uninfected H9 cells in an antagonistic manner. This conclusion must be drawn for the 50% inhibitory dose (ID_{50}) obtained from dose–response experiments (i) for each drug alone (ID_{50} for Avarol, 1.9 ± 0.2 μM and for AZT, 1.6 ± 0.1 μM), and (ii) for combinations of the two compounds (Fig. 2A). Presenting the data in the form of an isobologram results in a curve to the right of an imaginary straight line joining the ID_{50} concentration for single drug application, e.g. at the drug combination 0.75 μM of AZT and 1.48 μM of Avarol the FIC value was estimated to be 1.25, indicating significant antagonism.

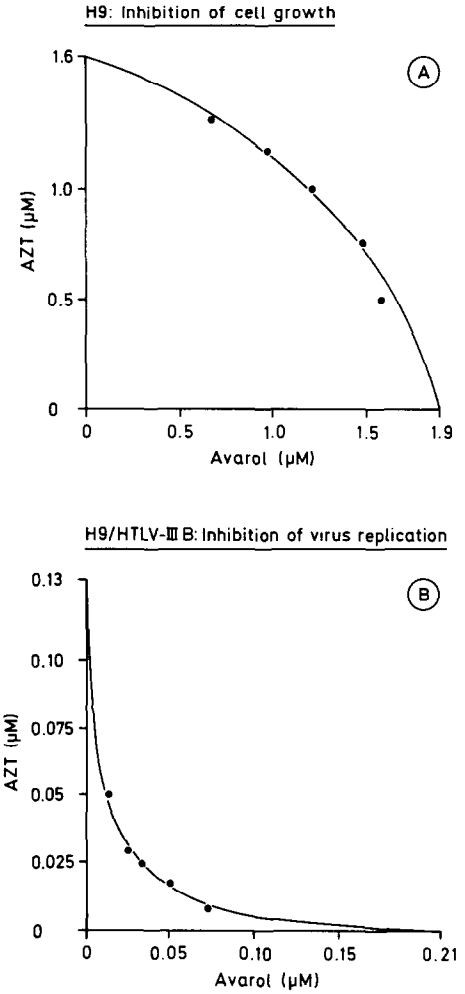

Fig. 2. Interactions that result from the combination of Avarol with AZT on (i) growth of uninfected H9 cells (A) and (ii) virus replication in HTLV-IIIB-infected H9 cells (B). (A) Uninfected H9 cells were incubated in the standard assay for 4 days and the inhibitory dose required for 50% inhibition of cell growth (ID_{50}) was determined for each compound alone; ID_{50} for Avarol: 1.9 ± 0.2 μM and for AZT: 1.6 ± 0.1 μM. Then the ID_{50} concentrations for selected combinations of AZT and Avarol were determined (●) and plotted according to the isobologram procedure. (B) H9 cells were infected with HTLV-IIIB and incubated for 4 days in the absence of any drug, in the presence of Avarol or AZT, or a combination of these two drugs. Then the supernatants were collected after centrifugation and subjected to the reverse transcriptase assay. The concentrations required for 50% inhibition of virus replication by a single drug were determined by dose–response experiments and the ED_{50}'s were estimated as follows; Avarol: 0.21 ± 0.02 μM and AZT: 0.13 ± 0.02 μM. The 50% inhibition value, obtained for a combination of selected drug concentrations, are indicated (●) and plotted in a form of an isobologram.

The conclusions of the combination experiments are twofold. (i) Avarol and AZT influence HIV replication by different modes of action in H9 cells. This result is not surprising in view of the above mentioned influence of AZT on reverse transcriptase and of Avarol on viral protease. (ii) The two compounds inhibit cell growth at a 9-fold (for Avarol) and 12-fold (for AZT) higher concentration compared with the doses which effectively interfere with HIV growth. Most notable, given in combination, the antiviral effect of the two compounds is even more pronounced than the cytostatic effect caused by them. This conclusion must be drawn from the synergistic effect of the compounds on HIV growth and the antagonistic influence on cell proliferation. At present it can be suggested that the cytotoxic effect of AZT is due to an interaction with the cellular DNA polymerase alpha and the one of Avarol is caused at least partially by an influence on the selection mechanism(s) at the posttranscriptional level [33].

To ascertain if the observed selective inhibitory influence of Avarol and AZT on HIV formation with respect to their effects on cell growth also could be confirmed on a molecular level, quantitative measurements of viral and cellular mRNA levels were accomplished by RNA dot-blot hybridization and densitometric analysis. As a typical cellular gene we have selected the cytoskeletal β-actin gene, because it codes for one of the most abundant cellular proteins [34]; the HTLV-IIIB *pol* segment served as a probe to quantify viral gene expression. We found (to be published) that at the respective ED_{50} concentration of Avarol and AZT required for 50% inhibition of virus replication the expression of *pol* mRNA decreased to about 50%, as estimated by dot-blot hybridization. On the other hand, the level of cellular β-actin mRNA remains unchanged. This indicates that both drugs at concentrations protecting the cells against the cytopathic effects of HIV-1 do not affect cellular mRNA synthesis. In addition, the expression of the cellular interleukin-2 (IL-2) and interferon-γ (IFN-γ) genes was studied; the synthesis of IL-2 mRNA has previously been shown to be induced by *tat*-II protein in HTLV-II-infected Jurkat T-cells [35]. On the other hand, antigen-induced IL-2 secretion by T-cells from AIDS patients has been found to be impaired [36]. We found (to be published) that the concentration of IL-2 mRNA was significantly increased (to about 180%) in cells treated with Avarol plus AZT both present at half their ED_{50} concentration. Each drug alone, at its respective ED_{50} concentration, produced no significant increase in the amount of cellular IL-2 mRNA. No synthesis of IFN-γ mRNA could be detected in both uninfected and HTLV-IIIB-infected H9 cells. The lack of induction of IFN-γ gene expression in H9 cells by HTLV-IIIB has already been demonstrated previously [37].

Altered mRNA levels could result from changes in mRNA synthesis and stability, post-transcriptional processing, nucleocytoplasmic transport and degradation. To assess whether cytosolic factors are playing a dominant role in determining the amount of viral RNA released from HIV-1-infected cells as compared to cellular RNA, the influence of cytosolic proteins from HTLV-IIIB-infected H9 cells on the transport of cellular (actin, IL-2 and IFN-γ) and virus-encoded (*pol*) mRNAs from HTLV-IIIB/H9 cell nuclei was studied. Previously we reported [19] that in uninfected cells one of the cytosolic mRNA-transport-stimulatory proteins, p58, inhibits the nuclear envelope protein kinase that down-regulates the nuclear envelope nucleoside triphosphatase (NTPase) [38], the enzyme that mediates nucleocytoplasmic mRNA transport [39]. The other protein, p31, enhances the poly(A) binding affinity of the mRNA binding site in the envelope with resulting stimulation of nuclear envelope phosphoprotein phosphatase and NTPase [19].

Transport of viral and cellular mRNA was measured by dot-blot hybridization of the RNA released into the postnuclear supernatant and densitometric scanning of the autoradiograms using a standard curve (not shown). As shown in Fig. 3, addition of total cytosolic protein from HTLV-IIIB-infected H9 cells resulted in a differential effect on the efflux of cellular and viral mRNA. In the presence of 6 mg/ml of the dialyzed 100,000 *g* supernatant of a HTLV-IIIB/H9 cell homogenate [containing a series of virus-encoded proteins including p14 (11) *tat*-III [6, 40, 41] as checked by Western blotting using a positive patient's serum], a 7-fold stimulation of the nuclear export of viral *pol* mRNA

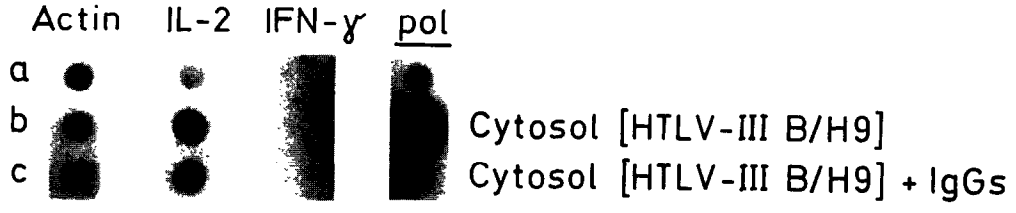

Fig. 3. Effect of total, cytosolic protein from HTLV-IIIB-infected H9 cells on efflux of cellular (actin, IL-2, and IFN-γ) and virus-encoded (*pol*) mRNAs from HTLV-IIIB/H9 cell nuclei. After an incubation of the cells for 4 days nuclei were isolated and RNA transport assays were performed in the absence (a) or presence of 6 mg/ml of total cytosolic protein from HTLV-IIIB-infected H9 cells (100,000 *g* supernatant of an H9 cell homogenate, dialyzed against 50 mM Tris–HCl pH 7.6, 2.5 mM $MgCl_2$, 25 mM KCl), that had been preincubated for 60 min at 22° without (b) or with (c) IgGs (purified by precipitation in 37% ammonium sulfate) from AIDS patient. The cellular and viral mRNA in the total postnuclear supernatant from 4×10^5 nuclei after a 30-min incubation period was analyzed by dot-blot hybridization.

Table 1. Efflux of actin mRNA and *pol* mRNA from nuclei of untreated and AZT- or Avarol-treated HTLV-IIIB/H9 cells in the absence or presence of cytosolic proteins from HTLV-IIIB/H9 cells

	Actin			*pol*		
	−	+AZT	+Avarol	−	+AZT	+Avarol
No addition	100	102	98	100	<5	<5
Cytosol [HTLV-IIIB/H9]	136	135	132	760	<5	<5
plus purified IgGs	134	137	135	175	<5	<5

After an incubation period of 4 days, nuclei were isolated from untreated, HTLV-IIIB-infected H9 cells or from cells grown in the presence of 0.1 μM AZT or 0.14 μM Avarol. RNA transport assays were performed for 30 min in the absence or presence of 6 mg/ml of total cytosolic protein from HTLV-IIIB-infected H9 cells (preincubated for 60 min at 22° without or with purified IgGs from serum of an AIDS patient). The actin mRNA and *pol* mRNA in the efflux supernatants were quantitated by dot-blot hybridization and densitometric scanning. The amount of actin mRNA and of *pol* mRNA released in the absence of cytosolic protein from untreated nuclei was set at 100%.

was obtained, while cellular β-actin mRNA transport increased only 1.4-fold. Interestingly, the efflux of IL-2 mRNA from isolated nuclei of HTLV-IIIB/H9 cells, induced with phytohemagglutinin (PHA) and 12-*O*-tetradecanoylphorbol 13-acetate (TPA) [37], was enhanced 2-fold (Fig. 3). This result confirms previous findings [37]. An LTR-linked expression of IL-2 has been shown also in HTLV-II-infected Jurkat T-cells [35]. No nuclear export of IFN-γ mRNA could be detected in the presence of HTLV-IIIB/H9 cell cytosol. Addition of purified IgGs from serum of an AIDS patient suppressed the stimulatory potency of the viral cytosolic proteins on efflux of *pol* mRNA and IL-2 mRNA to values found for the efflux of actin mRNA. From these results we conclude that virus-encoded cytoplasmic factors, most likely proteins [6, 19, 40], modulate the efflux of viral mRNA from nuclei *in vitro* and most likely also *in vivo*. In some cases, viral cytoplasmic factors seem also to be involved in regulation of synthesis and release of cellular mRNA, e.g. IL-2 mRNA.

Table 1 summarizes the effects of AZT and Avarol (at the respective ED_{50} concentration) on actin mRNA and *pol* mRNA efflux from isolated HTLV-IIIB/H9 cell nuclei. At their antiviral doses, both drugs displayed no effect on actin mRNA efflux, in the absence as well as in the presence of HTLV-IIIB/H9 cell cytosol. In the presence of the drugs, only a small amount of *pol* mRNA was exported out of the nuclei.

In conclusion, our results demonstrate that the cytoplasm of HIV-1 infected cells contains factors which are able to stimulate selectively the export of viral mRNA from cell nucleus *in vitro*. One of the HIV-1 encoded proteins synthesized in the cytoplasm is *tat*-III [6, 40]. It has been suggested by some authors [5, 6] that *tat* promotes the transcription of viral mRNA but does not significantly affect the level of viral mRNA, while others [42] showed increased mRNA levels. However, it is unknown whether these increased levels of HIV-1 mRNAs are due to an increased stability of viral mRNA or to an increased rate of initiation of transcription [43]. From the results presented in this paper also an enhanced nucleocytoplasmic transport could be responsible for altered viral mRNA levels. However, there are no hints that viral *tat*-III protein is involved in modulating RNA efflux rate. There is evidence also for the existence of additional factors that play a modulatory role in transcription, processing, transport and translation of viral mRNA [5, 44, 45].

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