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Inhibition of the *in vitro* infectivity and cytopathic effect of human T-lymphotrophic virus type III/lymphadenopathy-associated virus (HTLV-III/LAV) by 2′,3′-dideoxynucleosides

(acquired immunodeficiency syndrome)

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ABSTRACT **Human T-lymphotropic virus type III (HTLV-III)/lymphadenopathy-associated virus (LAV) is a newly discovered lymphotropic retrovirus that is cytopathic for helper/inducer T cells *in vitro*. This virus is the etiologic agent of the acquired immunodeficiency syndrome and related diseases. In the current study, we tested the capacity of purine and pyrimidine nucleoside derivatives to inhibit the infectivity and cytopathic effect of human T-lymphotropic virus type III *in vitro*. With the ribose moiety of the molecule in a 2′,3′-dideoxy configuration, every purine (adenosine, guanosine, and inosine) and pyrimidine (cytidine and thymidine) nucleoside tested suppressed the virus, although the thymidine derivative seemed to have substantially less activity in our system than the others. In general, we observed essentially complete suppression of the virus at doses that were lower by a factor of 10 to 20 than those needed to inhibit the proliferation of the target T cells and the immune reactivity of normal T cells *in vitro*. An analysis of five adenosine congeners, which differed only in the sugar moiety, revealed that reduction (an absence of hydroxyl determinants) at both the 2′ and 3′ carbons of the ribose was necessary for an anti-viral effect, and an additional reduction at the 5′ carbon nullified the anti-viral activity. These observations may be of value in developing a new class of experimental drugs for the therapy of human T-lymphotropic virus type III infections.**

Human T-lymphotropic virus type III (HTLV-III)/lymphadenopathy-associated virus (LAV) is a lymphotropic retrovirus which is cytopathic for helper/inducer T cells *in vitro* (1–7). This virus is the etiologic agent of the acquired immunodeficiency syndrome (AIDS) and related diseases (8, 9). To date, over 14,000 cases of AIDS have been reported in the United States; the incidence and prevalence of this disease continue to increase. AIDS is almost always fatal within 1 to 2 years after the first clinical manifestations of illness. This disease was initially described and characterized in four high-risk groups (homosexual men, hemophiliacs, Haitians, and intravenous drug abusers); however, individuals belonging to no apparent high-risk groups have developed the disease. AIDS is generally spread by intimate sexual contact or by the administration of blood products, and occasionally by the maternal–fetal route. Many patients who develop AIDS are asymptomatic when they transmit their disease to contacts because a 6-month to 5-year (or more) latency interval may exist between infection and clinical manifestations of illness (10).

Although a number of anti-viral agents are now being considered for the experimental therapy of AIDS (11–17), to date no therapy has been shown to cure HTLV-III/LAV infection or restore the underlying immunodeficiency. Moreover, the chronicity of infection (8, 10) and the propensity of the virus to infect the brain (18, 19) make it necessary to explore new classes of drugs that have the potential for oral administration and penetration across the blood-brain barrier. In the current study, we report that with the ribose moiety of the molecule in a 2′,3′-dideoxy configuration, every purine (adenosine, guanosine, and inosine) and pyrimidine (cytidine and thymidine) nucleoside tested suppressed the infectivity and the cytopathic effect of HTLV-III/LAV *in vitro* at concentrations that do not affect the growth and immune functions of T cells.

MATERIALS AND METHODS

HTLV-III/LAV Virus. HTLV-III_B was obtained from the culture supernatant of HTLV-III_B-producing H9 (H9/HTLV-III_B) cells as described (1). The virus was prepared to contain approximately 6×10^{10} virus particles per ml. The number of virus particles were determined by electron microscopy. In some experiments, irradiated (10,000 rad; 1 rad = 0.01 Gy) H9/HTLV-III_B cells were used as a source of infectious virions.

Nucleosides. 2′,3′-Dideoxyadenosine, 2′-deoxyadenosine, 2′,3′-dideoxyinosine, 2′,3′-dideoxycytidine were purchased from Calbiochem-Behring, while 2′,3′-dideoxyguanosine and 2′,3′-dideoxythymidine were purchased from Pharmacia. 3′-Deoxyadenosine (cordycepin) was purchased from Boehringer-Mannheim. 9-β-D-Arabinofuranosyladenine and 2′,3′,5′-trideoxyadenosine were generously provided by D. Johns, J. Driscoll, and G. Milne.

Cells. We have described the method for generating normal tetanus toxoid-specific T-cell clones (20), and one such normal $OKT4^+$ clone (TM3) was used in this study. Characteristics of clone TM3 have been described elsewhere (17). An immortalized $OKT4^+$ T-cell clone (ATH8) was obtained by cloning a normal tetanus-toxoid-specific T-cell line in the presence of human T-lymphotropic virus type I (HTLV-I)-producing cells as described (20). Clone H9 is an $OKT4^+$ T-cell line that is permissive to HTLV-III/LAV replication but partially resistant to the HTLV-III/LAV cytopathic effect as described by Popovic *et al.* (1).

HTLV-III/LAV Cytopathic Effect Assay. HTLV-III/LAV cytopathic effect assays were performed as described (11). ATH8 cells were used as target T cells without antigen stimulation. TM3 cells were stimulated by antigen plus irradiated (4000 rad) fresh autologous peripheral blood mononuclear cells in complete medium [RPMI 1640 supplemented

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Abbreviations: AIDS, acquired immunodeficiency syndrome; HTLV-I, human T-lymphotropic virus type I; HTLV-III, human T-lymphotropic virus type III; LAV, lymphadenopathy-associated virus.

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with 4 mM L-glutamine, 50 μM 2-mercaptoethanol, 15% (vol/vol) undialyzed heat-inactivated fetal calf serum, 50 units of penicillin/ml, and 50 μg of streptomycin per ml] containing 15% (vol/vol) interleukin 2 (lectin-depleted; Cellular Products, Buffalo, NY) 4 days before assay. After preexposure to 2 μg of polybrene (Sigma) per ml for 30 min, the target T cells (2×10^5) were pelleted, exposed to HTLV-III_B virus (2000 virus particles per cell) for 45 min, resuspended in 2 ml of fresh medium, and incubated in culture tubes (Falcon 3030) at 37°C in 5% CO_2/95% air humidified atmosphere. Control cells were treated similarly but were not exposed to the virus. In the coculture experiments, 4×10^5 lethally irradiated (10,000 rad) H9/HTLV-III_B cells were cultured with 2×10^5 target T cells in the presence or absence of 2′,3′-dideoxynucleosides. At various time points, the total viable cells were counted in a hemocytometer under the microscope by the trypan blue dye exclusion method.

Determination of HTLV-III/LAV gag Protein Expression. H9 cells (10^5) were exposed to various concentrations of 2′,3′-dideoxynucleosides for 4 hr and then to polybrene (2 μg/ml) for 30 min, pelleted, and exposed to HTLV-III_B virus (3000 virus particles per cell) for 1.5 hr. Cells were resuspended in fresh complete medium at 37°C in 5% CO_2/95% air humidified atmosphere. The cells were continuously exposed to the nucleosides. On days 8, 9, and 10 in culture, the percentage of the target H9 cells expressing p24 gag protein of HTLV-III/LAV was determined by indirect immunofluorescence microscopy as described (11, 17) by using anti-HTLV-III/LAV p24 murine monoclonal antibody (M26; kindly provided by F. D. Veronese and R. C. Gallo) (21).

Antigen- or Mitogen-Induced T-Cell Activation Assays. Washed responder TM3 cells (5×10^4) were cultured for 3 days with tetanus toxoid and 10^5 irradiated (4000 rad) autologous peripheral blood mononuclear cells in 160 μl of complete medium in round-bottom microtiter culture plates. In some experiments, 10^5 fresh peripheral blood mononuclear cells were cultured with or without polyclonal mitogens (phytohemagglutinin or pokeweed mitogen) for 3 days in flat-bottom microtiter culture plates. All cultured cells were exposed to 0.5 μCi (1 Ci = 37 GBq) of [methyl-H^3]thymidine or 1 μCi of [5-^{3}H]uridine for the final 5 hr and harvested onto glass fibers, and the incorporated radioactivity was counted.

RESULTS

Generation of HTLV-III/LAV Cytopathic Effect-Sensitive T-Cell Clone, ATH8. To perform these studies, we employed an HTLV-III/LAV-sensitive OKT4$^+$ T-cell clone (ATH8) suitable for rapid drug screening. This clone was selected for this study on the basis of its rapid growth (in the presence of interleukin 2) and readily apparent sensitivity to the cytopathic effect of the virus. Clone ATH8 bears several distinct copies of HTLV-I in its genome when assessed by Southern blot hybridization using a radiolabeled HTLV-I cDNA probe but does not produce detectable amounts of HTLV-I gag proteins (unpublished results).

In our system, HTLV-III/LAV (as cell-free virus) exerts a profound cytopathic effect on the target T cells by day 4 in culture, and by day 10, >98% of cells have been killed by the virus (Fig. 1*a*). The killing of cells can be monitored quantitatively as a function of the starting dose of virus particles (Table 1). When ATH8 cells were used in a 7-day assay, 5 virus particles per cell represented the minimum cytopathic dose of virus. In the experiments reported below, 2000 or 3000 virus particles per cell were used to test various compounds under conditions of substantial virus excess.

Protection of Helper/Inducer T Cells by 2′,3′-Dideoxynucleosides Against HTLV-III/LAV Cytopathic Effect. Fig. 1 (*b–e*) and Fig. 2 show the potent protective effect of 2′,3′-dideoxynucleosides on the survival and growth of ATH8 cells when exposed to HTLV-III/LAV. We found that concentrations of >10 μM 2′,3′-dideoxyadenosine, 2′,3′-dideoxyguanosine, and 2′,3′-dideoxyinosine, as well as concentrations of >0.5 μM 2′,3′-dideoxycytidine completely protected ATH8 cells and enabled them to survive and grow. These compounds exhibited a strong anti-viral effect at doses that were lower by a factor of 10 to 20 than those that inhibited growth of the target cells when no virus was present. Under our experimental conditions, 2′,3′-dideoxythymidine required relatively high concentrations to exert a protective effect, and unlike the other comparable dideoxynucleosides tested, its capacity to nullify the cytopathic effect of the virus was lost on day 10 of the culture (Fig. 1*e* and Fig. 2, *Bottom*).

The protective effects of 2′,3′-dideoxyadenosine, -inosine, and -cytidine were confirmed in normal helper/inducer TM3 cells that were cocultured with irradiated H9/HTLV-III_B cells (Fig. 3). When cultured in the absence of the nucleosides, by day 14, H9/HTLV-III_B cells exerted a substantial cytopathic effect on the TM3 population, resulting in a profound decrease in the number of total viable TM3 cells as compared to the virus-unexposed TM3 populations. However, 2′,3′-dideoxyadenosine (100 μM), -inosine (100 μM), and -cytidine (5 μM) clearly blocked the cytopathic effect of HTLV-III_B.

Inhibition of HTLV-III/LAV p24 gag Protein Expression in H9 Cells by 2′,3′-Dideoxynucleosides. These anti-viral effects were confirmed in a different system by using the expression of the HTLV-III/LAV p24 gag protein in H9 cells (Fig. 4). The H9 cells are relatively resistant to the cytopathic effect of HTLV-III/LAV, and one can use p24 gag protein expression following exposure to HTLV-III/LAV virions as an index of viral infectivity and replication *in vitro* (1). The 2′,3′-dideoxyderivatives of adenosine, guanosine, inosine, and cytidine were potent inhibitors of viral replication in this system, while as in the previous experiments, 2′,3′-

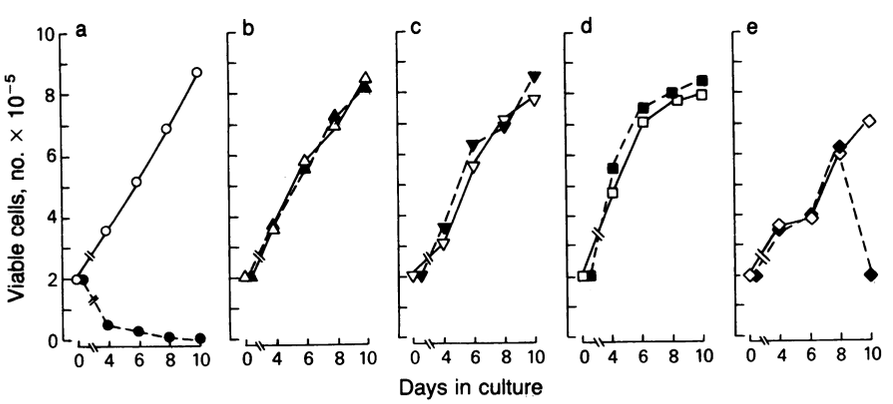

FIG. 1. Survival and growth of ATH8 cells in the presence of 2′,3′-dideoxynucleosides, when exposed to HTLV-III/LAV. ATH8 cells (2×10^5) were treated with polybrene, exposed to HTLV-III_B (2000 virus particles per cell), resuspended, and cultured in culture tubes (*a*). Solid symbol denotes the virus-exposed population while open symbol denotes the virus-unexposed control population. In the comparable experiments, ATH8 cells were continuously exposed to 50 μM 2′,3′-dideoxyadenosine (*b*), 50 μM 2′,3′-dideoxyinosine (*c*), 1 μM 2′,3′-dideoxycytidine (*d*), and 500 μM 2′,3′-dideoxythymidine (*e*). At various time points, total viable cells were counted.

Table 1. Cytopathic effect of HTLV-III/LAV on ATH8 cells

HTLV-III_B virus particles per cell	Viable ATH8 cells ($\times 10^5$)
0	3.37 ± 0.1
0.05	3.36 ± 0.04
0.5	3.26 ± 0.15
5	1.97 ± 0.2
50	1.78 ± 0.16
500	0.37 ± 0.02
5000	0.30 ± 0.01

ATH8 cells (10^5) were preexposed to polybrene for 30 min, pelleted, exposed to various amounts of HTLV-III_B, resuspended in 2 ml of interleukin 2-containing complete medium, and incubated in culture tubes at 37°C in 5% CO_2/95% air humidified atmosphere. On day 7, the total viable cells were counted by the trypan blue dye exclusion method. Data are expressed as the arithmetic means ± 1 standard deviation for duplicate determinations.

dideoxythymidine required relatively higher concentrations to mediate an anti-viral effect, and this compound allowed a resumption of viral replication by day 10 of culture (Fig. 4, *Bottom*).

Protection of ATH8 Cells by Adenosine Congeners Against the HTLV-III/LAV Cytopathic Effect. We then explored the capacity of five closely related congeners (2′,3′-dideoxyadenosine, 2′-deoxyadenosine, 3′-deoxyadenosine, 9-β-D-arabinofuranosyladenine, and 2′,3′,5′-trideoxyadenosine) to reverse the cytopathic effect of HTLV-III/LAV. The structure of each congener and its capacity to protect target ATH8 cells against the cytopathic effect of the virus are shown in Fig. 5. The congeners differed only in their sugar moieties. In these experiments, the 2′,3′-dideoxyadenosine completely protected the target ATH8 cells against lysis by the virus during the course of the culture. The other compounds failed to inhibit the cytopathic effect of the virus. The adenosine arabinoside did not mediate an anti-viral effect even when 2′-deoxycoformycin was added (data not shown).

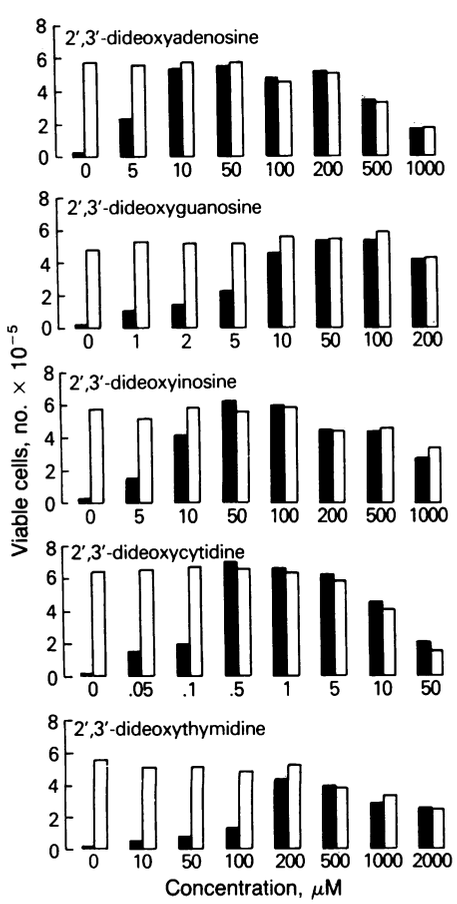

FIG. 2. Inhibition of cytopathic effect of HTLV-III/LAV by 2′,3′-dideoxynucleosides against ATH8 cells. ATH8 cells (2×10^5) were preexposed to polybrene, exposed to HTLV-III_B (2000 virus particles per cell) in culture tubes (solid columns) in the presence or absence of various concentrations of 2′,3′-dideoxyadenosine, -inosine, -guanosine, -cytidine, or -thymidine. Control cells (open columns) were similarly treated, but were not exposed to the virus. On day 5, total viable cells were counted.

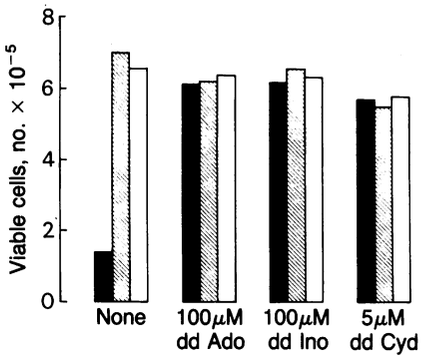

FIG. 3. Protection of a normal helper/inducer T-cell clone TM3 when cocultured with HTLV-III_B-producing H9 cells by 2′,3′-dideoxynucleosides. TM3 cells (2×10^5) were exposed to polybrene, and cocultured with 4×10^5 irradiated HTLV-III_B-producing H9 cells (solid columns) or uninfected H9 cells (hatched columns) in the presence or absence of 2′,3′-dideoxynucleosides. Control TM3 cells (open columns) were cultured without any cells added. When cultured alone, none of irradiated HTLV-III_B-producing H9 cells or uninfected H9 cells were alive on day 6 in culture. TM3 cells could be readily distinguished from neoplastic H9 cells by morphology. On day 14, total viable cells were counted. dd Ado, 2′,3′-dideoxyadenosine; dd Ino, 2′,3′-dideoxyinosine; dd Cyd, 2′,3′-dideoxycytidine.

2′,3′-Dideoxynucleosides Do not Inhibit Functions of T Cells at Concentrations that Block Replication and Cytopathic Effect of HTLV-III/LAV. We then tested the effects of the various 2′,3′-dideoxynucleosides on the antigen-specific and lectin-induced reactivity of normal lymphocytes *in vitro* (Table 2). We used a normal helper/inducer T-cell clone TM3 to monitor the effects of the compounds on antigen-driven activation, and normal circulating lymphocytes to monitor the effects on phytohemagglutinin- and pokeweed mitogen-driven activation. Concentrations of these compounds including those that were 10- to 20-fold higher than those necessary to block the *in vitro* infectivity and cytopathic effect of HTLV-III/LAV left the *in vitro* immune reactivity of normal T cells basically intact.

DISCUSSION

The data reported here indicate that any 2′,3′-dideoxynucleoside tested has the potential to function as an anti-viral agent against the replication of HTLV-III/LAV. (In the case of 2′,3′-dideoxyinosine, the anti-viral effect could theoretically be mediated by a salvage pathway conversion to either 2′,3′-dideoxyadenosine or 2′,3′-dideoxyguanosine.) As the 2′,3′-dideoxynucleosides are successively phosphorylated to yield 2′,3′-dideoxynucleoside-5′-triphosphates, they become analogues of 2′-deoxynucleotides that are the natural substrates for cellular DNA polymerases and viral reverse transcriptases. In this context, the lack of activity against HTLV-III/LAV that we found using 2′,3′,5′-trideoxyadenosine (Fig. 5*e*) is very likely a consequence of the unavailability of the 5′ site to undergo phosphorylation.

The mechanisms by which 2′,3′-dideoxynucleosides suppress the replication of HTLV-III/LAV are not known. It is known that the 5′-triphosphate product of 2′,3′-dideoxyaden-

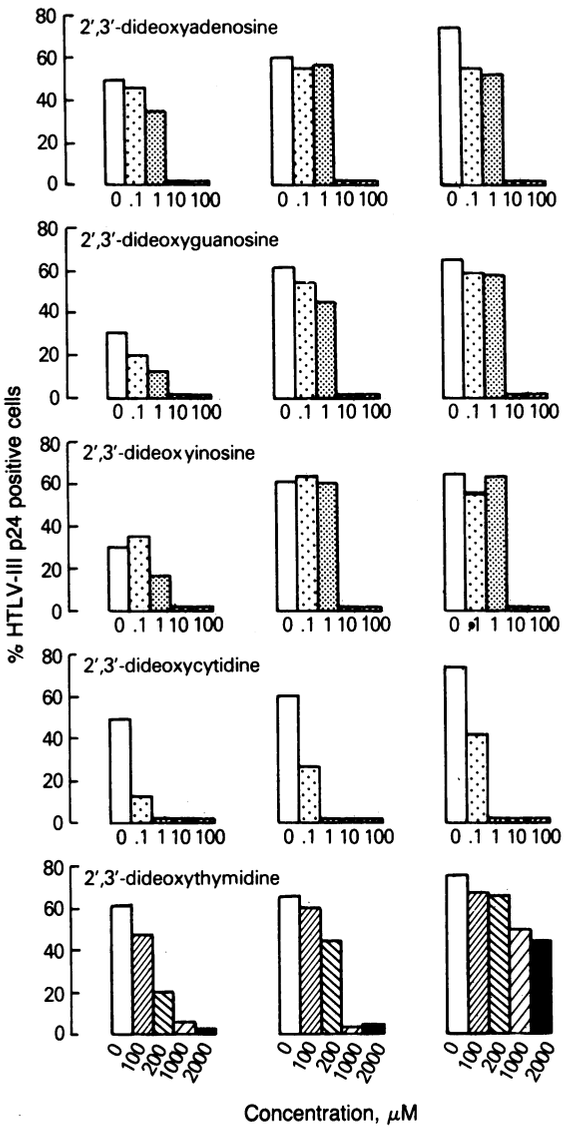

FIG. 4. Inhibition of the infectivity and replication of HTLV-III/LAV in H9 cells by 2',3'-dideoxynucleosides. H9 cells (10^5) were exposed to various concentrations of 2',3'-dideoxynucleosides for 4 hr and then to polybrene, pelleted, and exposed to HTLV-III_B (3000 virus particles per cell). Cells were resuspended in fresh complete medium and cultured in test tubes. The cells were continuously exposed to 2',3'-dideoxynucleosides. On days 8 (*Left*), 9 (*Middle*), and 10 (*Right*) in culture, the percentage of the target H9 cells expressing p24 gag protein of HTLV-III/LAV was determined by indirect immunofluorescence assay using a murine monoclonal antibody (M26).

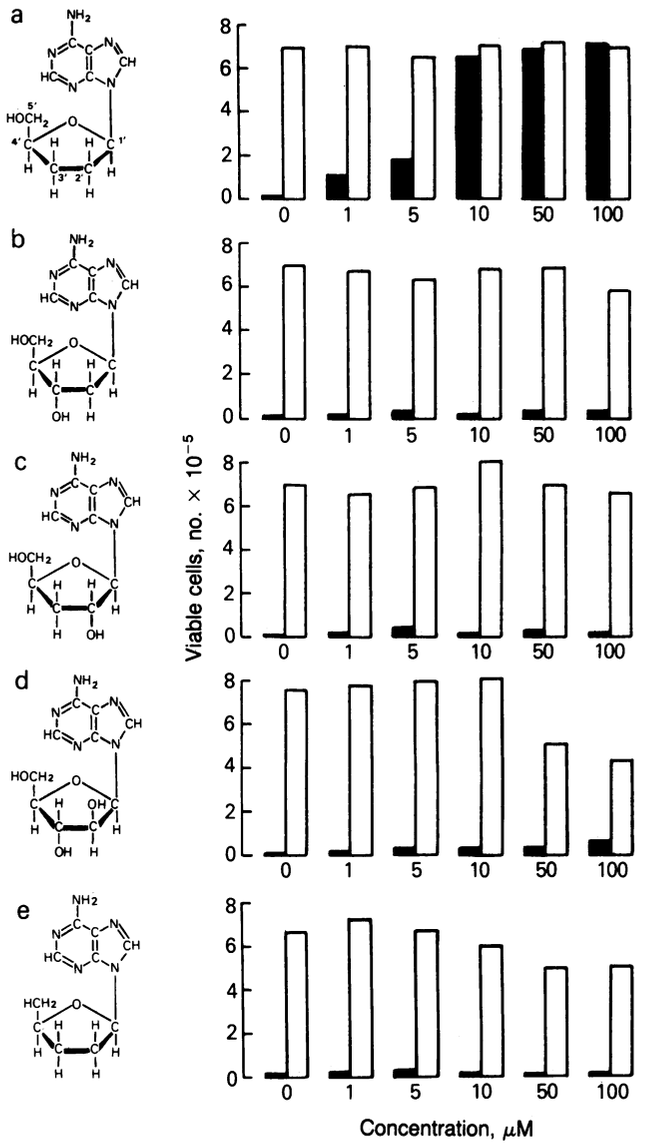

FIG. 5. Protection of ATH8 cells against the cytopathic effect of HTLV-III/LAV by adenosine congeners. ATH8 cells (2×10^5) were preexposed to polybrene, exposed to HTLV-III_B (2000 virus particles per cell), resuspended in culture tubes (solid columns) in the presence or absence of various amounts of adenosine congeners: 2',3'-dideoxyadenosine (*a*), 2'-deoxyadenosine (*b*), 3'-deoxyadenosine (cordycepin) (*c*), and 9-β-D-arabinofuranosyladenine (*d*), and 2',3',5'-trideoxyadenosine (*e*). The primed numbers in *a* refer to positions in the sugar moiety. Control cells (open columns) were not exposed to the virus. On day 5, the total viable cells were counted by dye exclusion method.

osine, -dideoxyguanosine, -dideoxycytidine, and -dideoxythymidine can rather easily inhibit cellular DNA polymerases β and γ, as well as viral reverse transcriptase (*vide infra*), but not mammalian DNA polymerase α(22–26). DNA polymerase α is assumed to be the key DNA synthetic enzyme for DNA replication during cell division, and it also has a role in DNA repair (26–28). Of interest, Herpes simplex type I DNA polymerase is reported to be as resistant to 2',3'-dideoxythymidine as cellular DNA polymerase α (29). It is worth stressing that the four (unphosphorylated) dideoxynucleosides have rather negligible effects on the growth of cultured mammalian cells (26) (a phenomenon that we confirm here in human T cells). This is probably so because of inefficient intracellular conversion to the corresponding 5' triphosphates coupled with the resistance of DNA polymerase α to low levels of the 5' triphosphates (26).

Of special interest in view of the experiments reported here, 2',3'-dideoxythymidine-5'-triphosphate also inhibits reverse transcriptases from type C RNA tumor viruses (30, 31), and the 2',3'-dideoxynucleosides have been shown to inhibit the replication of mouse retroviruses in mouse (and human) cell lines (26, 32), but not in rat and chicken cell lines (30, 32), presumably because the latter lack appropriate kinases required for conversion of the nucleoside to an active, phosphorylated molecule. The inhibition of reverse transcriptase probably occurs by the incorporation of a dideoxynucleotide at the 3' end of growing chains of unintegrated viral DNA, thereby terminating the elongation of DNA chains at the point where the dideoxynucleotide is substituted for the normal nucleotide since a phosphodiester linkage at the 3' position of the sugar cannot occur (33). However, the dideoxynucleosides under discussion could be

Table 2. Effect of 2′,3′-dideoxynucleosides on the *in vitro* immune reactivity of normal lymphocytes

Responder cells	None	Adenosine*		Guanosine*		Inosine*		None§	Cytidine§		Thymidine§	
		10	100	10	100	10	100		1	10	200	2000
Clone TM3†	75 ± 6	86 ± 8	67 ± 2	66 ± 5	77 ± 1	62 ± 4	81 ± 4	12 ± 1	13 ± 1	8 ± 1	9 ± 1	9 ± 1
PBM + PHA‡	310 ± 39	298 ± 18	314 ± 37	350 ± 4	469 ± 9	298 ± 20	355 ± 37	82 ± 1	101 ± 1	86 ± 3	86 ± 6	77 ± 8
PBM + PWM‡	63 ± 2	63 ± 5	57 ± 3	73 ± 8	62 ± 5	61 ± 1	62 ± 5	24 ± 1	23 ± 3	15 ± 3	31 ± 1	31 ± 1

*All nucleosides tested are of 2′,3′-dideoxy configuration. Micromolar concentrations of each 2′,3′-dideoxynucleoside bring about virtually complete inhibition of the cytopathic effect of HTLV-III/LAV (Figs. 1 and 2) and viral p24 expression (except 2′,3′-dideoxythymidine; see Fig. 4). [^{3}H]Thymidine incorporation was used as an indicator of activation of responder cells, unless otherwise indicated.

†Normal helper/inducer TM3 cells (5×10^4) were stimulated with tetanus toxoid (0.6 limiting flocculation units/ml) plus 10^5 irradiated autologous PBM and cultured for 72 hr in the presence or absence of the nucleosides. Cells were exposed to [^{3}H]thymidine for the final 5 hr, harvested onto glass fibers, and the incorporated radioactivity was counted. Data are expressed as the arithmetic mean counts per minute ($\times 10^3$) ± 1 standard deviation of triplicate determinations.

‡PBM (10^6) from a healthy individual were stimulated with PHA or PWM and cultured for 72 hr and treated as described above. PBM, peripheral blood mononuclear cells; PHA, phytohemagglutinin; PWM, pokeweed mitogen.

§[^{3}H]Uridine incorporation was used as an indicator of activation of the responder cells.

converted to metabolites that differ from the expected phosphorylation products (26), and therefore, one must consider the possibility that the effects reported here are mediated by as yet unidentified metabolites. Moreover, it is possible that the various dideoxynucleosides might not all be metabolized in the identical way in virally infected and uninfected cells.

Regardless of the mechanism(s), the results reported here demonstrate that a simple chemical modification of the sugar moiety can predictably convert a normal substrate for nucleic acid synthesis into a potent compound with the capacity to inhibit the replication and cytopathic effect of HTLV-III/LAV. These results might have implications in the development of new strategies for the pharmacologic intervention against pathogenic human retroviruses *in vivo*.

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