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3′-Azido-3′-deoxythymidine (BW A509U): An antiviral agent that inhibits the infectivity and cytopathic effect of human T-lymphotropic virus type III/lymphadenopathy-associated virus *in vitro*

(inhibition of human T-lymphotropic virus type III/thymidine analogue/acquired immune deficiency syndrome/retrovirus)

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ABSTRACT **The acquired immune deficiency syndrome (AIDS) is thought to result from infection of T cells by a pathogenic human retrovirus, human T-lymphotropic virus type III (HTLV-III) or lymphadenopathy-associated virus (LAV). In this report, we describe the antiviral effects of a thymidine analogue, 3′-azido-3′-deoxythymidine (BW A509U), which, as a triphosphate, inhibits the reverse transcriptase of HTLV-III/LAV. This agent blocks the expression of the p24 *gag* protein of HTLV-III/LAV in H9 cells following exposure to virus. The drug also inhibits the cytopathic effect of HTLV-III$_B$ (a virus derived from a pool of American patients) and HTLV-III/RF-II (an isolate obtained from a Haitian patient that differs by about 20% in the amino acid sequence of the envelope gene from several isolates of HTLV-III/LAV, including HTLV-III$_B$, analyzed so far). 3′-Azido-3′-deoxythymidine also completely blocks viral replication as assessed by reverse transcriptase production in normal human peripheral blood mononuclear cells exposed to HTLV-III$_B$. Finally, at concentrations of 3′-azido-3′-deoxythymidine that block the *in vitro* infectivity and cytopathic effect of HTLV-III$_B$, the *in vitro* immune functions of normal T cells remain basically intact.**

The acquired immune deficiency syndrome (AIDS) is a disease characterized by extensive immunosuppression that predisposes patients to life-threatening opportunistic infections and unusual forms of neoplasms (1–7). Several converging lines of investigation have clearly linked a cytopathic retrovirus, human T-lymphotropic virus type III (HTLV-III) (8–11) or lymphadenopathy-associated virus (LAV) (12–14) to the pathogenesis of AIDS. This virus preferentially infects and destroys OKT4$^+$ (helper/inducer) T-cells. Previously, Mitsuya *et al.* have shown that suramin, a drug used in the therapy of African trypanosomiasis and onchocerciasis and known to inhibit reverse transcriptase of animal retroviruses (15), can block *in vitro* infectivity and cytopathic effect of HTLV-III/LAV at concentrations that do not adversely affect the *in vitro* immune functions of lymphocytes (16, 17). Suramin also can inhibit the replication of HTLV-III/LAV *in vivo* (unpublished data). At present, only a few other compounds are immediate candidates as experimental drugs to inhibit HTLV-III/LAV replication—e.g., antimoniotungstate (HPA-23) (18), ribavirin (19), recombinant human α interferon (20), ansamycin,¶ and phosphonoformic acid (21). To date no experimental regimen has been proven to restore the underlying immunodeficiency of the disease, and essentially all drugs under study may have substantial side effects. Thus, there is a crucial need to develop new agents and therapeutic strategies. In the present study, we report the capacity of the antiviral agent 3′-azido-3′-deoxythymidine (BW A509U) to inhibit HTLV-III/LAV replication and to block the cytopathic effect of HTLV-III/LAV *in vitro* under a variety of conditions.

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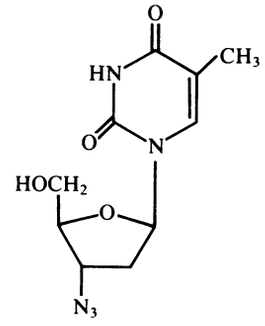
MATERIALS AND METHODS

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 previously described (8). The virus was prepared to contain approximately 10^{10} virus particles per ml. In some experiments, irradiated (10,000 rads; 100 Gy) H9 cells, producing a Haitian variant of HTLV-III/LAV(HTLV-III/RF-II) (8), were used as a source of infectious virions. The Haitian variant differs from the several closely related virus genotypes in H9/HTLV-III$_B$ cells by about 20% in the amino acid sequence of its envelope gene (22).

Nucleoside Analogue. A nucleoside analogue, 3′-azido-3′-deoxythymidine (BW A509U) (M_r 267.24), was synthesized as described by Lin and Prusoff (23). This compound (structure I) is referred to as A509U in this paper.



Structure I

The compound is a competitive inhibitor of the reverse transcriptase of HTLV-III/LAV as a triphosphate, but the

Abbreviations: AIDS, acquired immune deficiency syndrome; Con A, concanavalin A; HTLV-I, human T-lymphotropic virus type I; HTLV-III, human T-lymphotropic virus type III; IL-2, interleukin-2; LAV, lymphadenopathy-associated virus; PBM, peripheral blood mononuclear cells; PHA, phytohemagglutinin.

¶Anand, R., Moore, J. L., Srinivason, A., Kalyanaraman, V., Francis, D., Feorino, P. & Curran, J., Abstracts of International Conference on Acquired Immune Deficiency Syndrome (AIDS), April 14–17, 1985, Atlanta, GA, p. 72.

unphosphorylated compound does not inhibit reverse transcriptase *per se* (unpublished data).

Cells. Clone H9 is an $OKT4^+$ T-cell line that is permissive to HTLV-III/LAV replication but partially resistant to its cytopathic effect as previously described (8).

We have previously described the method for generating normal tetanus-toxoid-specific T-cell clones (24), and one such clone (TM3) was used in these studies. An immortalized T-cell clone (ATH8) was obtained by cloning a normal tetanus-toxoid-specific T-cell line in the presence of lethally irradiated (12,000 rads; 120 Gy) human T-lymphotropic virus type I (HTLV-I)-producing MJ-tumor cells (25). Clone ATH8 was selected for this study on the basis of its rapid growth (in the absence of antigen) and exquisite sensitivity to the cytopathic effect of $HTLV\text{-}III_B$ (unpublished data).

Determination of HTLV-III/LAV *gag* Protein Expression. H9 cells (2×10^5) were exposed to various concentrations of A509U for 4 hr and then to Polybrene (2 μg/ml; Sigma) for 30 min, pelleted, and exposed to $HTLV\text{-}III_B$ virus (1000 virions per cell) for 1.5 hr. Cells were resuspended in fresh complete medium (RPMI 1640 medium supplemented with 4 mM L-glutamine, 50 μM 2-mercaptoethanol, 15% undialyzed, heat-inactivated fetal calf serum, and 50 units of penicillin and 50 μg of streptomycin per ml) and were cultured in tubes (3033, Falcon) at 37°C in 5% CO_2-containing humidified air. The cells were continuously exposed to A509U. 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 (8, 16) by using anti-HTLV-III/LAV p24 murine monoclonal antibody (M26) (26).

Inhibition Assay for the Cytopathic Effect of HTLV-III/LAV. HTLV-III/LAV cytopathic effect inhibition assays were performed as described (16). Briefly, clone TM3 cells were stimulated by antigen plus irradiated (4000 rads; 40 Gy) fresh autologous peripheral blood mononuclear cells (PBM) and were cultured in complete medium containing 15% (vol/vol) interleukin 2 (IL-2, lectin-depleted; Cellular Products, Buffalo, NY) 6 days before assay. ATH8 cells were used without the antigen stimulation. After preexposure to 2 μg of Polybrene per ml for 30 min, the target T cells (2×10^5) were pelleted, exposed to $HTLV\text{-}III_B$ virus for 45 min, resuspended in 2 ml of fresh medium, and incubated in culture tubes at 37°C in 5% CO_2-containing humidified air. Control cells were treated similarly but were not exposed to the virus. The cells were exposed continuously to IL-2 and A509U. When ATH8 cells were used in this assay system, five virus particles per cell were the minimum cytopathic dose of virus (unpublished data). In the cell coculture experiments, 5×10^4 lethally irradiated (10,000 rads) HTLV-III/RF-II-producing H9 cells or uninfected H9 cells were added to 2×10^5 target T cells. At various time points, the total viable cells were counted in a hemocytometer under the microscope by the trypan blue dye exclusion method.

Assay for Reverse Transcriptase. PBM (10^6) from a healthy individual that had been cultured for 7 days after phytohemagglutinin (PHA)-stimulation were exposed to $HTLV\text{-}III_B$ and cultured in the presence and absence of various concentrations of A509U. On the following day, cells were washed, resuspended in fresh medium, and cultured further with the same concentrations of the agent. On day 5 in culture, supernatants were harvested and subjected to reverse transcriptase assay as described (8, 9). Results are expressed as counts per minute of deoxy[*methyl*-^{3}H]thymidine triphosphate (40–60 Ci/mmol; 1 Ci = 37 GBq) incorporated per 10 μl of concentrated (25-fold) culture supernatants.

Antigen- or Mitogen-Induced T-Cell Activation Assays. Washed responder TM3 cells (4×10^4) were cultured for 3 days with 0.6 limiting flocculation units/ml of tetanus toxoid and 8×10^4 irradiated (4000 rads) autologous PBM in 160 μl of 15% fetal calf serum-containing complete medium in round-bottom microtiter culture plates. In some experiments, 10^5 fresh PBM were cultured with or without polyclonal mitogen [PHA or concanavalin A (Con A)] for 3 days in flat-bottom microtiter culture plates. All cultured cells were exposed to 0.5 μCi of [5-^{3}H]thymidine (25.8 Ci/mmol) for the final 5 hr and harvested onto glass fibers. The incorporated radioactivity was assayed.

Immunoglobulin Production and Helper Assays. Indicator B cells (consisting of B cells and monocytes) were separated from PBM from the normal individual from whom clone TM3 was derived by using neuraminidase-treated sheep erythrocytes as described (27). Indicator B cells (10^5) were exposed to tetanus toxoid (2 limiting flocculation units/ml) for 4 hr, extensively washed, and cocultured with 5×10^4 TM3 cells in 0.3 ml of 15% fetal calf serum-containing complete medium. On day 8 the culture supernatants were harvested and assessed for IgG production by using an enzyme-linked immunosorbent assay (28).

RESULTS

Inhibition of HTLV-III/LAV p24 *gag* Protein Expression in H9 Cells by A509U. We first addressed the inhibitory effect of A509U on the expression of HTLV-III/LAV p24 in clone H9, a lymphoblastoid line, that is permissive for HTLV-III replication (8). When the target H9 cells were exposed to the $HTLV\text{-}III_B$ isolate (1,000 virus particles per cell) and cultured in the absence of A509U, by day 10, 74% of the target H9 cells became infected and expressed p24 *gag* protein as determined by an indirect immunofluorescence assay (Fig. 1). A striking protective effect was observed when the H9 cells were cultured in the presence of 1 μM A509U. A complete protective effect was observed at concentrations of 5 and 10 μM, and none of H9 cells became positive for p24 throughout the 10-day interval of culture.

Protection of Helper/Inducer T Cells by A509U Against HTLV-III/LAV Cytopathic Effect. We then asked whether A509U could block the cytopathic effect of $HTLV\text{-}III_B$ against a normal IL-2-dependent helper/inducer T-cell clone, TM3. This clone displays the following surface membrane antigen phenotype: $OKT3^+$, $OKT4^+$, Tac-antigen$^+$, $HLA\text{-}DR^+$, and $OKT8^-$. Clone TM3 undergoes a proliferative

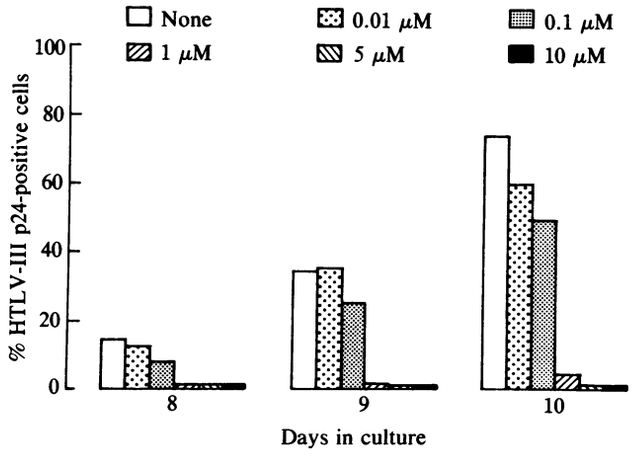


FIG. 1. Inhibition of expression of HTLV-III/LAV p24 in H9 cells by A509U. Clone H9 cells were cultured in the presence and absence of various concentrations of A509U after exposure to $HTLV\text{-}III_B$ in the form of cell-free virions. At various times, the percentage of H9 cells expressing HTLV-III/LAV p24 *gag* gene product was assessed by indirect immunofluorescence microscopy with anti-HTLV-III/LAV p24 murine monoclonal antibody.

reaction in response to the specific antigen, tetanus toxoid, in the presence of appropriate accessory cells. In addition, TM3 shows helper activity for immunoglobulin production by normal B cells as discussed later.

Five thousand virus particles per cell were used to produce a cytopathic effect of HTLV-III$_B$ in the target T cells. Fig. 2 *Upper* illustrates the protective effect of A509U on the survival and growth of clone TM3 when exposed to HTLV-III$_B$. Since TM3 cells had been stimulated by soluble tetanus toxoid 6 days before, these cells continued to grow in the presence of exogenous IL-2. In the absence of the drug, the HTLV-III$_B$ virions exerted a substantial cytopathic effect on the TM3 population by day 10 in culture, resulting in a profound decrease in the number of total viable cells, as compared with the control HTLV-III$_B$-unexposed TM3 population. However, the addition of 5 or 10 μM A509U protected TM3 cells and enabled them to survive and grow.

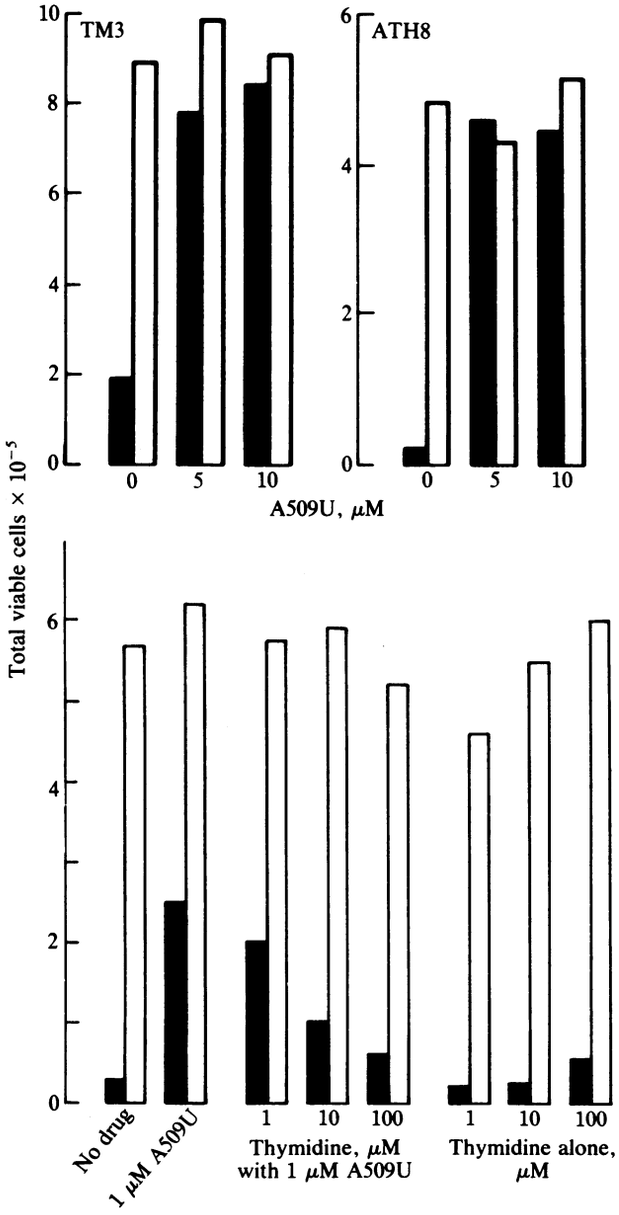


FIG. 2. Inhibition of cytopathic effect of HTLV-III$_B$ by A509U against helper/inducer T cells and reversal of the inhibitory effect of A509U by thymidine. (*Upper*) Protective effect of A509U against clones TM3 and ATH8 exposed to HTLV-III$_B$. TM3 and ATH8 cells (2×10^5) were exposed to HTLV-III$_B$ and cultured in the presence of 0, 5, and 10 μM A509U (■). Control cells were similarly treated, but were not exposed to the virus (□). On days 10 (TM3) or 7 (ATH8), the total viable cells were counted. (*Lower*) Reversal of the protective effect of A509U by thymidine. ATH8 cells (2×10^5) were exposed to HTLV-III$_B$ and cultured in the presence and absence of 1 μM A509U and/or 1 to 100 μM thymidine (■). On day 8 in culture, the total viable cells were counted. Similarly treated control cells were not exposed to virus (□).

The protective effects of the drug were confirmed in clone ATH8. Clone ATH8 bears several distinct copies of HTLV-I in its genome but does not produce HTLV-I p24 *gag* protein (unpublished data). The surface phenotype of this clone is OKT3$^+$, OKT4$^+$, Tac$^+$, HLA-DR$^+$, and OKT8$^-$. When cultured in the absence of A509U, by day 7 after HTLV-III$_B$ exposure, almost all ATH8 cells were killed (Fig. 2 *Upper*). The susceptibility of this clone to the HTLV-III$_B$ cytopathic effect was much greater than that of clone TM3. However, the addition of 5 and 10 μM of A509U could again completely protect ATH8 cells against the cytopathic effect of HTLV-III$_B$.

Reversal of A509U Inhibition of HTLV-III/LAV Cytopathic Effect by Thymidine. We attempted to test the possibility that A509U inhibits the HTLV-III/LAV cytopathic effect by acting as a competitive analog of thymidine. When exposed to HTLV-III$_B$ (5000 virus particles per cell) together with 1 μM A509U, ATH8 cells were moderately protected against the virus (Fig. 2 *Lower*). The addition of thymidine reversed the protective effect of A509U, and the target ATH8 cells were lysed in a dose-dependent fashion. When added alone, thymidine did not exert any cytotoxic effect.

A509U Protects the Target T Cells Against the Cytopathic Effect of a Haitian Isolate of HTLV-III/LAV (HTLV-III/RF-II). To determine whether A509U is effective against a genetically different isolate of HTLV-III/LAV, we tested the effect of the drug against HTLV-III/RF-II, a variant of HTLV-III/LAV isolated from a Haitian patient with AIDS showing significant divergence in its genome from other isolates of HTLV-III/LAV (22). The results shown in Fig. 3 illustrate the protective effect of A509U on the survival and growth of the clone ATH8 after exposure to HTLV-III/RF-II. In the absence of A509U, lethally irradiated HTLV-III/RF-II-producing H9 cells exerted a substantial cytopathic effect on the ATH8 population. Most of the target cells were killed by day 5 in culture. We found that 0.5 μM A509U showed a partial protective effect of the cells against the cytopathic effect. However, at concentrations of 1 to 10 μM,

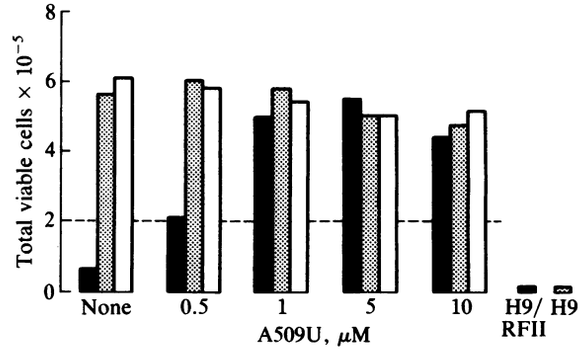


FIG. 3. Inhibition of cytopathic effect exerted by HTLV-III/RF-II (a Haitian isolate)-producing H9 cells against ATH8 cells by A509U. ATH8 cells (2×10^5) were cocultured with 5×10^4 lethally irradiated HTLV-III/RF-II-producing H9 cells (H9/RF-II) (■) or uninfected H9 cells (▨) in the presence of various concentrations of A509U. Control cells were cultured without any cells added (□). On day 5 in culture, the total viable cells were counted. ATH8 cells could be readily distinguished from neoplastic H9 cells by morphology. On day 5, none of the irradiated HTLV-III/RF-II-producing H9 or irradiated uninfected H9 cells cultured alone were found alive. The dashed horizontal line shows the starting number of ATH8 cells.

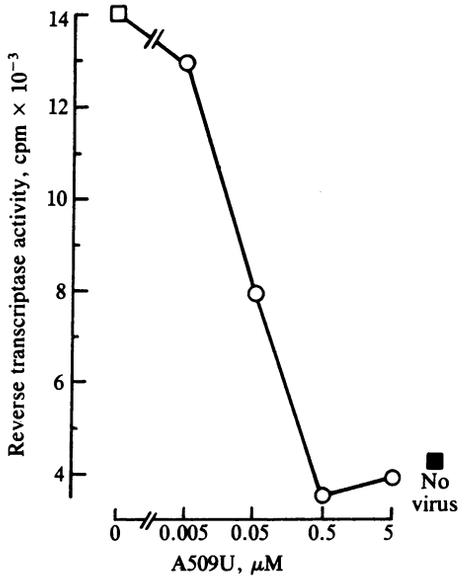


FIG. 4. Inhibition of A509U in reverse transcriptase production by PBM exposed to HTLV-III$_B$. PBM which had been cultured for 7 days after PHA-stimulation were exposed to HTLV-III$_B$ and cultured in the presence or absence of various concentrations of A509U. On the following day, cells were washed, resuspended in fresh medium, and cultured further with the same concentration of A509U. On day 5 in culture, supernatants were collected after centrifugation and subjected to the reverse transcriptase assay.

A509U clearly blocked the cytopathic effect of HTLV-III/RF-II. These data might also suggest that A509U is able to block HTLV-III/LAV infection caused by both extracellular and cell-associated virions.

Inhibition of Reverse Transcriptase Production by HTLV-III$_B$-Exposed PBM. To extend our observations, we investigated whether the addition of A509U inhibits reverse transcriptase production in normal PBM exposed to HTLV-III$_B$. As shown in Fig. 4, a substantial level of reverse transcriptase activity could be detected in the supernatants of normal PBM exposed to HTLV-III$_B$ in the absence of A509U. However, addition of A509U resulted in a dose-related decrease in reverse transcriptase activity in the supernatants. Inhibition was observed at doses as low as 0.005 μM and was marked at 0.05 μM. Complete inhibition was achieved at doses of 0.5 μM and more.

A509U Does Not Inhibit Functions of T and B Cells at Concentrations That Block Replication and Cytopathic Effect of HTLV-III/LAV. We tested the effects of A509U on the *in vitro* immunologic functions of the helper/inducer T-cell clone TM3 and PBM from normal individuals. A509U exerted a slight inhibitory effect on the antigen-induced response of TM3 at a concentration of 1 μM. At a concentration of 50 μM, T-cell activation was only partially inhibited (Fig. 5*A*). The mitogen-induced activation of PBM using PHA or Con A was only marginally inhibited at concentrations up to 10 μM (Fig. 5*B*). When cocultured with tetanus toxoid-preexposed indicator B cells (consisting of autologous B cells and monocytes), TM3 cells showed a significant level of helper activity in inducing IgG production by the indicator B cells (Fig. 5*C*). This immunoglobulin production was partially inhibited at a concentration of 50 μM of A509U; however, it was not inhibited in a dose range of 1–10 μM, indicating that A509U at these concentrations exerts only moderate inhibitory effects on immunoglobulin production by B cells in this system of helper activity. When assessed by trypan blue dye exclusion, the viability of TM3 cells that had been cultured for 14 days in the presence of various concentrations of A509U (0 to 10 μM) was approximately the same and not adversely affected (Fig. 5*D*). These data suggest that, at concentrations that block the *in vitro* infectivity and cytopathic effect of HTLV-III/LAV, A509U only slightly inhibits some immunologic reactivities of normal T cells and that a substantial level of *in vitro* immune reactivity is preserved, even at relatively large doses (up to 50 μM).

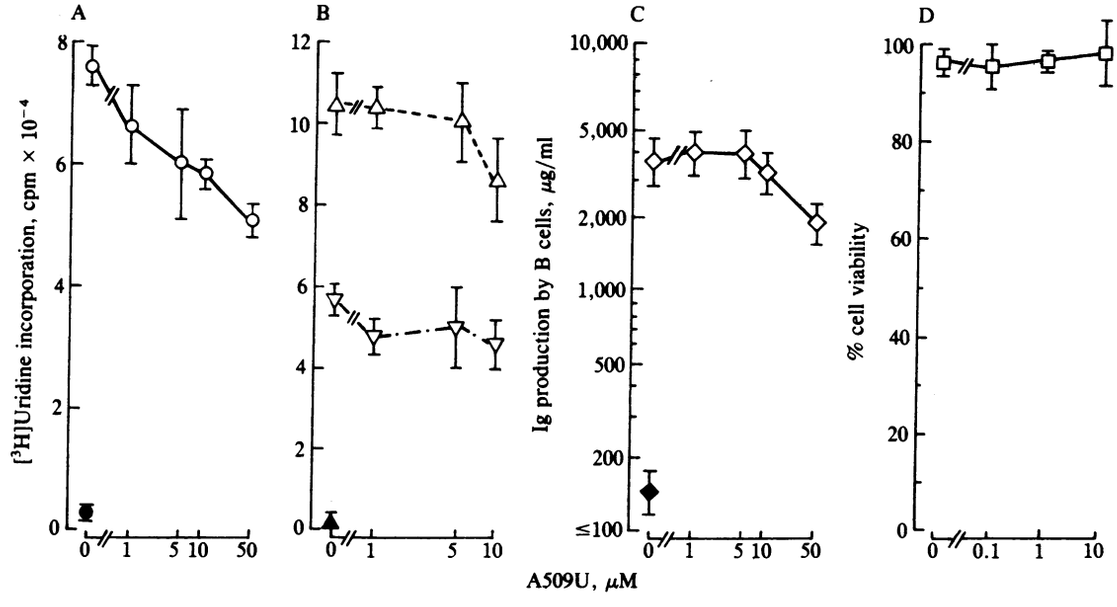


FIG. 5. Effect of A509U on the antigen- or mitogen-induced activation and immunoglobulin production. Various amounts of A509U were added to 5 × 10^4 TM3 cells that were stimulated with tetanus toxoid plus 10^5 irradiated autologous PBM (*A*), 10^5 PBM from a healthy individual that were stimulated with PHA (△) or Con A (▽) (*B*), or 10^5 tetanus toxoid-preexposed indicator B cells that were cocultured with 5 × 10^4 TM3 cells (*C*). The antigen- or mitogen-induced activation of clone TM3 and PBM was assessed by incorporation of [^{3}H]uridine into cellular RNA, whereas the helper activity of TM3 cells was assessed by IgG production by indicator B cells. Each solid symbol denotes the background [^{3}H]uridine incorporation or IgG production when the responder cells were cultured alone in the absence of A509U, antigen, or mitogen. (*D*) Viability of TM3 cells when cells were exposed to various concentrations of A509U and 15% IL-2 for 14 days, with the viability assessed by trypan blue exclusion. Data are expressed as the means ± 1 SD of triplicate determinations.

DISCUSSION

It is worth stressing that the activity of an agent against viruses *in vitro* does not ensure that the agent will be clinically useful in treating viral diseases. Toxicity, metabolic features, bioavailability, and other factors could negate the clinical utility of a given agent. Moreover, it is possible that patients with the most advanced forms of AIDS might have an immunodeficiency state that no longer depends on HTLV-III/LAV replication and, therefore, would require therapeutic interventions beyond an antiviral agent *per se*. Nevertheless, there are certain characteristics that would argue for cautious exploration of A509U as an experimental drug in patients with HTLV-III/LAV infection. First, the drug produces little or no toxicity even when given in high doses (85–150 mg/kg of body weight) in rats and dogs for up to 4 weeks (K. Ayers, personal communication). Second, the drug can bring about essentially complete inhibition of viral replication at doses that do not substantially diminish various *in vitro* parameters of T-cell immune reactivity. Finally, from experience with other nucleoside analogues, it is likely that A509U will be absorbed by oral administration, making it potentially suitable for regimens that involve prolonged therapy. Taken together, we believe these features provide a rationale for considering this drug as an experimental antiviral agent in certain patients with HTLV-III/LAV infection.

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