Effect of Several Antiviral Agents on Human Lymphocyte Functions and Marrow Progenitor Cell Proliferation

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Toxicity to hematopoiesis and lymphocytic function are major considerations in the clinical applicability of antiviral agents. We have examined the toxicities of five antiviral agents showing activity against herpesviruses: vidarabine, acyclovir, (*E*)-5-(2-bromovinyl)-2'-deoxyuridine, trifluorothymidine, and (*S*)-9-(2,3-dihydroxypropyl)adenine. The drugs were tested in vitro for inhibition of herpes simplex virus type 1 and human cytomegalovirus replication, effects on marrow progenitor cell growth, effects on lymphocyte responses to mitogen and alloantigen stimulation, and effects on several lymphocyte cytotoxic responses. In general, lymphocyte proliferative responses were inhibited by the various drugs at lower concentrations than were cytotoxic activities. Acyclovir and (*E*)-5-(2-bromovinyl)-2'-deoxyuridine were the least toxic drugs tested, with antiviral indices exceeding 10,000. Vidarabine and trifluorothymidine were more toxic, with antiviral indices generally between 10 and 100. (*S*)-9-(2,3-dihydroxypropyl)adenine was the most toxic, with several antiviral indices between 1 and 10.

Several antiviral agents have demonstrated in vitro efficacy against herpes simplex virus (HSV) or cytomegalovirus (CMV) and hold promise for the treatment of herpesvirus infections in humans. Vidarabine, acyclovir (ACV), and (*E*)-5-(2-bromovinyl)-2'-deoxyuridine (BVdU) are nucleoside analogs which are active against HSV and varicella-zoster virus (2, 9, 10, 25, 31). Trifluorothymidine (TFT) and (*S*)-9-(2,3-dihydroxypropyl)adenine (DHPA) are also nucleoside analogs with activity against HSV and CMV (9, 13, 30, 32). Hematopoietic toxicity has been the main dose-limiting factor in the clinical use of antiviral agents. There is also concern that such agents may be immunosuppressive in situations where immune responses may be crucial to recovery (5, 23). We have examined the effects of these five promising antiviral agents on human marrow progenitor cell proliferation and various lymphocyte functions in vitro.

MATERIALS AND METHODS

Drugs. ACV and TFT were generously supplied by Burroughs Wellcome Co. (Research Triangle Park, N.C.). DHPA was obtained from Calbiochem (La Jolla, Calif.). BVdU was a gift from E. DeClercq (Leuven, Belgium). Vidarabine was obtained from Parke, Davis & Co. (Morris Plains, N.J.).

Cells and antiviral assays. Mouse embryo fibroblasts and human foreskin fibroblasts were prepared and used as previously described (32). One-step replication assays for the AD169 strain of human CMV in human foreskin fibroblasts were conducted over 96 h, and

titters were determined as previously described (32). One-step replication assays for a clinical isolate of HSV type 1 in mouse embryo fibroblasts were conducted over 24 h, and titers were determined in Vero cells as previously described (6). The assay of anti-HSV activity of DHPA was conducted in Vero cells, since no anti-HSV activity was observed in mouse embryo fibroblasts. The multiplicity of infection (MOI) was 0.1 to 0.5 for both HSV and strain AD169. All assays were performed in quadruplicate and averaged, and standard errors were calculated.

Human bone marrow progenitor cell assay. The antiviral agents were tested for toxicity to human bone marrow stem cells by the soft agar assay for CFU-culture (CFU-c) as previously described (32). Briefly, marrow cells from normal donors at 2×10^5 cells per ml in supplemented McCoy 5A medium with 15% fetal calf serum and 0.3% agar were added in quadruplicate 1.0-ml portions to 35-mm petri dishes containing human placenta-derived colony-stimulating factor and various concentrations of the antiviral agents to be tested. After 10 days of incubation at 37°C in 7.5% CO₂, the number of colonies in drug-containing plates was scored, and the results were expressed as percentages of the number of colonies in control plates. Bone marrow cells from at least four normal subjects were used to assay each drug.

Lymphocyte proliferation assays. Peripheral blood lymphocytes were obtained from whole blood containing preservative-free sodium heparin by Ficoll-Hypaque gradient centrifugation and suspended at a concentration of 2×10^6 cells per ml in RPMI 1640 (GIBCO Laboratories, Grand Island, N.Y.) containing 20% pooled normal human serum, 1% glutamine, and 100 U of penicillin and 100 µg of streptomycin per ml.

Mixed lymphocyte reaction (MLR) cultures were established as previously described (12) by adding 2×10^5 responding lymphocytes in a volume of 0.1 ml and an equal volume of 2×10^5 irradiated (1,500 rads, ^{137}Cs source) stimulator cells to wells of flat-bottomed microtiter plates (Microtest II; Falcon Plastics, Oxnard, Calif.) in medium containing no drug or graded concentrations of the test drugs. All tests were done in triplicate, and the cultures were incubated at 37°C in 5% CO_2 . After 6 days of incubation, 1 μCi of [^3H]thymidine (New England Nuclear Corp., Boston, Mass.; specific activity, 6.7 Ci/mmol) was added to each well, and cultures were allowed to further incubate for 16 to 18 h before being harvested onto glass fiber filter paper (grade 934 AH; Reeve Angel, Clifton, N.J.). The filter papers were dried and processed for liquid scintillation counting. Results obtained as the mean counts per minute of [^3H]thymidine uptake $\pm$ the standard error of the mean were converted to percentages of control cultures with no drug.

Mitogen responses of peripheral blood lymphocytes were measured in a manner similar to that described for the MLR. Microcultures were established in flat-bottomed microtiter plates by adding 0.1 ml of cell suspension (2×10^5 lymphocytes) and 0.05 ml of medium containing graded quantities of the test drugs to each well. Either 1 μg of phytohemagglutinin (PHA) (Sigma Chemical Co., St. Louis, Mo.) or 5 μg of concanavalin A (ConA) in a volume of 0.05 ml was added to each well. On day 3 of the PHA cultures and day 4 of the ConA cultures, the plates were pulsed with 1 μCi of [^3H]thymidine per well, incubated for 16 to 18 h, and processed for liquid scintillation counting.

Cytotoxicity assays. To measure cell-mediated lympholysis (CML), 6×10^6 responding lymphocytes were cocultured with an equal number of irradiated (1,500 rads) stimulating lymphocytes in 6 ml of medium in 30-ml tissue culture flasks (Corning Tissue Culture Flask; Corning Glass Works, Corning, N.Y.) at 37°C . After 8 days of culture, primed cells were harvested from the MLR cultures and used as effectors in the CML assay. A total of 10^4 3-day-cultured, PHA-stimulated blast cells labeled with ^{51}Cr served as targets. Effector and target cells were incubated in the wells of round-bottomed microtiter plates (Linbro Chemical Co., New Haven, Conn.) in medium containing graded concentrations of drug in a final volume of 0.20 ml. Before incubation for 4 h at 37°C , the plates were subjected to light centrifugation ($100 \times g$, 1 min). At the end of the incubation period, the plates were centrifuged ($500 \times g$, 5 min), and 0.1 ml of the supernatant was removed and counted for released ^{51}Cr . Maximum and spontaneous ^{51}Cr release were obtained by adding 0.1 ml of 5% Triton X-100 (Sigma) in water or 0.1 ml of medium, respectively, to wells containing 2×10^4 target cells. The percent specific ^{51}Cr release was calculated according to the following formula: specific lysis = $[(\text{cpm experimental} - \text{cpm spontaneous})/(\text{cpm maximum} - \text{cpm spontaneous})] \times 100\%$. Spontaneous lysis values for all target cells with or without drug were between 15 and 30%.

Target cells for assay of natural killer (NK) activity were prepared from the K562 myeloid cell line (22). Antibody-dependent killer (K) cells were measured by an antibody-dependent cell-mediated cytotoxicity assay using antibody-sensitized Chang liver cells as target cells. This antibody was a gift from G. Quinnan,

Bethesda, Md., and was prepared in rabbits according to the method of Ortaldo et al. (19). Antibody-dependent cell-mediated cytotoxicity was determined by subtracting the lysis of unsensitized targets from that of sensitized targets. Target cells were labeled by suspension of 2×10^6 cells per ml in RPMI 1640 with 10% fetal calf serum containing 100 μCi of ^{51}Cr -sodium chromate (New England Nuclear) per ml for 90 min at 37°C , washed twice, and suspended in RPMI 1640 with 10% fetal calf serum at a concentration of 10^7 cells per ml. Cytotoxicity assays were performed in round-bottomed microtiter plates, using effector/target ratios of 25:1 and 12.5:1. Because the results obtained at the 25:1 ratio were representative, only these are presented. After an 18-h incubation at 37°C in a 5% CO_2 -air atmosphere, 0.1 ml of the supernatant was harvested and counted on a gamma counter. Specific lysis was calculated by using the equation given above. Assays were conducted in quadruplicate.

Analysis. The 50% inhibitory dose (ID_{50}) indicates the concentration of drug which reduced the test result to one-half of the control result and was obtained from semi-log plots of the virus yield (as a percentage of the control) against drug concentrations (on a log scale). Confidence intervals were calculated from the standard error of the estimate of the curve derived by linear regression analysis (4). The term antiviral index refers to the ratio of the ID_{50} against a host cell function to the antiviral ID_{50} .

RESULTS

The effects of the antiviral agents on virus replication, CFU-c, and various lymphocyte functions are given in Table 1. Both the antiviral and the anticellular effects of all of the antiviral agents were dose dependent. For all drugs tested, substantial activity against HSV type 1 or CMV was demonstrated at drug concentrations exerting little inhibition of hematopoietic growth and lymphocyte responses.

Both ACV and BVdU exhibited little toxicity to lymphocyte stimulation and marrow progenitor cell proliferation. The antiviral indices of both drugs exceeded 10,000. Vidarabine exhibited toxicity at much lower concentrations relative to the anti-HSV ID_{50} : 30- to 150-fold-higher doses were required for inhibition of CFU-c and mitogen stimulation than for anti-HSV activity, and 1,000- to 3,000-fold-higher doses inhibited CML and MLR. TFT was even more toxic to lymphocytes: stimulation by ConA, MLR, and CML was inhibited at concentrations 8- to 10-fold greater than the anti-CMV ID_{50} . However, CFU-c and stimulation by PHA were more resistant, being inhibited at doses more than 100-fold higher than the antiviral ID_{50} . NK and K cell activities were resistant to inhibition by TFT. DHPA was the most toxic drug tested: MLR and stimulation by ConA were inhibited at the anti-CMV ID_{50} . The CFU-c was inhibited at doses 10-fold higher than the antiviral ID_{50} . CML and NK and K cell activities were more

resistant to DHPA, with inhibition exceeding the antiviral ID₅₀ by greater than 10-fold.

DISCUSSION

The tests chosen to assay relative drug toxicity were directed toward a broad range of hematopoietic and lymphocytic cell functions. The CFU-c assay is a measure of the in vitro growth of human hematopoietic progenitor cells (granulocyte and macrophage progenitors). ConA and PHA are mitogens which induce proliferation primarily of T lymphocytes. The MLR measures the proliferation of helper and cytotoxic T cells after recognition of foreign antigens on other lymphocytes. CML represents killing of labeled target cells by sensitized T cells. NK cells are lymphocytes which do not express T or B cell surface markers but exhibit cytotoxic activity against tumor and virus-infected cells without prior sensitization. K cells are lymphocytes which also do not express T or B cell surface markers but have Fc receptors on their surface and demonstrate cytotoxicity against target cells sensitized with antibody.

Our results showed ACV and BVdU to be the least toxic drugs to hematopoietic and lymphocytic cells in these assays, with very large margins of predicted safety (antiviral indices exceeding 10,000). Both ACV and BVdU are preferentially phosphorylated by virus-encoded thymidine kinase and not by the cellular thymidine kinase. Once phosphorylated, both ACV and BVdU inhibit viral DNA polymerase to a greater extent than cellular DNA polymerase (10, 11). Thus, uninfected cells escape toxicity of the activated ACV and BVdU at concentrations far in excess of those demonstrating antiviral activity. The effects of ACV on CFU-c in our study are similar to those described in previous reports (18, 20). We observed inhibition of ConA and PHA stimulation by ACV at concentrations somewhat lower than those described in earlier reports (17, 27), which is probably attributable to variability of responsiveness between different donors (17) or to technical differences in these assays. Vidarabine and TFT demonstrated greater toxicity, with inhibition of several cell functions at antiviral indices generally between 10 and 100. Our results with vidarabine are consistent with those reported by Steele et al. (26), who found no inhibition of several lymphocytic functions at approximately 10 μ M. Of interest is the observation that vidarabine was more toxic to CFU-c than to the lymphocyte functions, whereas for TFT, several lymphocyte functions were more sensitive than CFU-c. DHPA was the most toxic drug tested, with several cellular functions inhibited at an index of 10 or less. DHPA has been found to have inhibitory activity against adenosine deaminase

TABLE 1. ID₅₀s of antiviral agents on virus replication, various lymphocyte functions, and marrow progenitor cell proliferation

Function tested	ID ₅₀ (μ M) ^a of:				
	Vidarabine	BVdU	ACV	TFT	DHPA
HSV	0.14 (0.10-0.16)	0.0029 (0.0019-0.0050)	0.0042 (0.0021-0.0071)	0.10 (0.079-0.13)	90 (80-160)
CMV	2.6 (1.2-5.0)	ND ^{b,c}	ND ^{b,d}	0.013 (0.010-0.016)	50 (25-130)
CFU-c	4.2 (1.5-5.0)	120 (79-160)	>100	1.3 (0.5-2.5)	560 (250-500)
MLR	460 (140-790)	450 (200-1,000)	120 (63-250)	0.11 (0.10-0.13)	26 (16-31)
ConA	8.6 (5.0-16)	270 (100-320)	52 (20-120)	0.13 (0.079-2.5)	45 (31-79)
PHA	>20	310 (240-390)	28 (8.0-89)	7.6 (5.0-16)	350 (10-790)
CML	190 (120-400)	>100	>100	0.24 (0.20-0.50)	>580
NK cell activity	>100	>100	>100	>100	>1,200
K cell activity	>100	>100	>100	>100	>1,200

^a ID₅₀ rounded off to two significant figures, with range encompassing the standard error given within parentheses.

^b ND, Not determined.

^c ID₅₀ > 750 μ M (E. De Clercq, personal communication).

^d ID₅₀ > 100 μ M (7).

(9), and adenosine deaminase deficiency has been noted in some patients with severe combined immunodeficiency (21). Several adenosine deaminase inhibitors have demonstrated profound lymphocytotoxic and immunosuppressive effects (29), and our findings are consistent with these earlier observations.

These results indicate that no single *in vitro* test is sufficient to predict the toxicity of drugs on hematopoietic and lymphopoietic cell function. Different drugs had variable effects in the different assays, despite similarities in the antiviral mechanism of action of some compounds. The NK and K cell functions appeared to be uniformly resistant to inhibition by the drugs at relevant concentrations and were not helpful in predicting drug toxicity. This is not surprising, since they, and the CML assay, test cytotoxic responses (effector cell functions) rather than proliferative responses and thus escape the primary effects of these drugs.

How well these and other similar *in vitro* assays predict *in vivo* toxicities is not clear at present. Certainly the lack of hematopoietic toxicity for ACV found in these assays fits well with clinical observations (15, 24). Likewise, clinical use of vidarabine and TFT has shown myelosuppression to be their limiting factor (1, 14, 16). Both BVdU and DHPA appear to be relatively nontoxic in *in vivo* rodent studies (9, 10). One preliminary study suggested no toxicity of BVdU in a small number of patients treated (8). Our tests raise the concern that DHPA may have hematopoietic and lymphocytic toxicity in human trials.

In addition, how well *in vitro*-derived antiviral ID₅₀s predict concentrations which are efficacious *in vivo* remains unclear at present. Three antiviral assay procedures are used widely: the plaque reduction test, the plaque inhibition test, and the yield reduction test (YRT). The plaque reduction test employs inoculation of a cell monolayer with a given number of viral PFU. After adsorption, agar containing no drug or graded concentrations of test drug is overlaid, and after an incubation period the resultant plaques are counted and expressed as a percentage of the virus control. The plaque inhibition test is similar, except paper disks are impregnated with the test drug and placed onto the agar overlay; the drug diffuses through the agar and produces a zone of inhibition of viral cytopathic effect, and the diameter of the zone is measured. A high MOI is used to ensure extensive cytopathic effect, which is important to produce as distinct a zone as possible. The YRT employs inoculation of cells with virus, and liquid medium containing no drug or graded concentrations of drug is added. After a single viral replication cycle the resultant virus yield at given concen-

trations of drug is expressed as a percentage of the virus control.

All three assays have disadvantages. The plaque inhibition test depends on the solubility and rate of diffusion of drugs in semisolid medium, the subjective assessment of zones of inhibition, and the availability of high-titer stock virus preparations (a major drawback with a virus such as human CMV). The plaque reduction test also requires a semisolid medium which cannot be readily changed, a major drawback with a slow-growing virus such as human CMV because depletion of nutrients as well as breakdown of the test drug can lead to underestimation of the activity of the drug. Since the YRT uses a liquid medium, the drug-containing medium can be changed without affecting the virus yield during the latent phase of viral replication, thus ensuring that fresh drug is present during the critical time of DNA synthesis. This advantage has led us to adopt the YRT as our standard assay of antiviral activity for a slowly replicating virus like human CMV. The MOI also influences the test result; the highest MOIs obtainable for human CMV were used in this study, and similar MOIs for HSV were used for comparability.

All of the antiviral ID₅₀s in this study were derived by using the YRT, thus permitting comparison of the various drugs with one another. Caution should be exercised when comparing the ID₅₀s of drugs in this study with results from other studies using different assay procedures. For example, several of the drugs in our study were tested against HSV type 1 in another study, which used the plaque reduction test, and were found to have higher ID₅₀s (24). Further experience is needed to determine which *in vitro* assay correlates best with *in vivo* efficacy.

It appears that recovery from herpesvirus infections requires at least some host immunity, especially cell-mediated immunity (5, 23). Antiviral agents which exhibit substantial hematopoietic or lymphocytic toxicity, such as 5-iodo-2'-deoxyuridine and cytarabine (3, 28), have not been efficacious in human trials despite *in vitro* antiviral activity. Tests of hematopoietic and lymphocytic functions, such as those used in this study, may prove to be useful in the preclinical evaluation of candidate antiviral agents.

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