Comparative In Vitro Immunotoxicology of Acyclovir and Other Antiviral Agents

RUSSELL W. STEELE,^{1*} DANIEL J. MARMER,¹ AND RONALD E. KEENEY²

Section of Immunology and Infectious Diseases, Department of Pediatrics, University of Arkansas for Medical Sciences, Little Rock, Arkansas 72205¹; Division of Microbiology and Immunology, Cell Biology Branch HFT 164, National Center for Toxicological Research, Jefferson, Arkansas 72079²; and Virology Section, Medical Division, Burroughs Wellcome Co., Research Triangle Park, North Carolina 27709³

In vitro lymphocyte blastogenic responses to the commonly employed mitogens phytohemagglutinin, pokeweed, and concanavalin A were evaluated when acyclovir, adenine arabinoside, cytosine arabinoside, and idoxuridine were added to the culture materials. Similarly, specific antigen-induced blastogenic responses, including herpes group antigens, and cytotoxicity and leukocyte inhibitory factor assays with herpes group viruses were determined in the presence and absence of antiviral agents. No depression of these cellular immune responses by acyclovir or adenine arabinoside was demonstrated. This was in contrast to the effects of cytosine arabinoside and idoxuridine, which severely inhibited blastogenic and cytotoxic responses but not leukocyte inhibitory factor production. Even at concentrations up to 20 $\mu\text{g/ml}$, the antiviral agent acyclovir did not depress selected cellular immune responses that are important for successful elimination of invading herpes group viruses.

The efficacy of antiviral chemotherapeutic agents is determined primarily by their ability to inhibit viral replication. However, it has become apparent in the clinical setting that successful eradication of certain viral infections requires some degree of host immune competence, especially when infection is severe (15). Therefore, it seems important that, while inhibiting viral replication, antiviral chemotherapeutic agents must not suppress host immunity below as-yet-undefined limits.

Defense against viral infection involves a complex interplay between humoral and cell-mediated immune responses, and although the relative contributions of the two have not been fully delineated, the severe or fatal infections in children with thymic deficiency syndromes or in patients on immunosuppressive therapy suggest that cellular immunity is certainly one of the major host factors in combating herpes group viral disease (16). Therefore, antiviral chemotherapy agents that unduly depress the host's cellular immune responses without complete eradication of the virus could result in relapse of disease upon withdrawal of therapy. Previous laboratory and clinical reports have suggested that those antiviral agents that produce significant immunosuppression (13) prove to be quite limited therapeutically and are potentially more dangerous than the disease itself (19).

A new antiviral agent, 9-(2-hydroxyethoxymethyl)guanine (acyclovir), has been investi-

gated for its activity against herpesviruses in vitro and in infected animal models (1, 5-12) and is currently being introduced into human clinical studies. Acyclovir has been demonstrated to have low toxicity for normal cells in vitro (4) and strong antiviral activity against members of the herpes group of viruses, with a safety index (concentrations associated with toxicity divided by therapeutic concentrations) in tissue culture of approximately 3,000 (11). In vivo efficacy of acyclovir has been demonstrated in several animal models when administered parenterally, orally, and as cutaneous and ophthalmic ointments (1, 5-12).

The studies discussed in this report were designed to seek possible immunotoxic effects of acyclovir on mechanisms of cellular immunity in comparison to other antiviral agents.

MATERIALS AND METHODS

Immunological assays. The following in vitro correlates of immune function were examined: (i) mitogen-induced blastogenesis; (ii) antigen-induced blastogenesis; (iii) direct lymphocyte cytotoxicity to MA-160 cells persistently infected with herpes group viruses; (iv) leukocyte inhibitory factor (LIF) production in response to herpes group viruses.

Isolation of lymphocytes. Lymphocytes were separated from peripheral whole blood by centrifugation on a Hypaque-Ficoll gradient as previously described (21). These lymphocytes were obtained from donors who were immune, and some who were not immune, to each of the herpes group viruses on each

experimental day to provide appropriate negative and positive controls.

Mitogen stimulation. Using a biopipette, 0.1 ml of the lymphocyte suspension (2×10^5 lymphocytes) was added to an equal volume of various concentrations of the mitogens phytohemagglutinin, pokeweed, and concanavalin A, with each in one well of sterile flat-bottom microtest plates. A harvesting apparatus, previously described and technically refined for our use, was employed for separation of the stimulated lymphocytes on glass-fiber filters, for washing of these cells, and for recovery of radioactive [^3H]thymidine uptake (14). The average counts per minute of triplicate samples were determined, and results were expressed as a blastogenic index (BI): counts per minute of [^3H]thymidine uptake for lymphocytes incubated with mitogen, divided by uptake after incubation with medium alone.

Cell cultures persistently infected with herpes group viruses. Cell line MA-160, derived from human prostate adenoma tissue, was inoculated with herpes simplex type 1 (HSV-1), HSV-2, varicella-zoster virus, or cytomegalovirus at a multiplicity of infection of 1. The development of the resulting infected cell lines is described in a separate communication (18). Both the control MA-160 and virus-infected cell lines were monitored before use for surface membrane antigen by indirect immunofluorescence. An average of 60 to 70% of the infected cells fluoresced. Control cells did not fluoresce.

Assays of antigen-induced lymphocyte blastogenesis. Blastogenic responses to *Candida albicans* (Dermatophytin "O", Hollister-Stier Laboratories, Inc., Yeadon, Pa.) and tetanus toxoid (Merrell-National Laboratories, Cincinnati, Ohio) were determined as previously described (17). Methods for herpes group virus-induced blastogenesis with infected MA-160 cells were similar to those employed for a one-way mixed lymphocyte culture (20). Simultaneous controls were run using uninfected MA-160 cells. Counts per minute for lymphocytes incubated with uninfected MA-160 cells showed very little variation throughout the study, averaging approximately 1,200. Therefore, observed changes in BIs could not be accounted for by changes in control cultures.

Assays of lymphocyte cytotoxicity. A ^{51}Cr microassay of lymphocytotoxicity to the cell lines persistently infected with HSV-1, HSV-2, or varicella-zoster virus was used in these studies. Details of the microassay were described previously (14). Briefly, this technique examines lymphocyte-target cell interaction, employing the infected cell lines described above as the target cells. The quantitative release of ^{51}Cr from these target cells is used as an index of lymphocyte-mediated cytotoxicity against the uninfected cells. Uninfected cells served as controls to quantitate ^{51}Cr release not attributable to the virus itself. Target cell suspensions were labeled with ^{51}Cr (Na^{51}Cr ; New England Nuclear Corp.; specific activity, 50 to 500 Ci/g) by incubating 10^7 cells in 1 ml of medium containing 100 μCi of ^{51}Cr for 45 min as described previously.

A sample of 0.1 ml of lymphocytes (5×10^5) was incubated with 0.1 ml of target cells (5×10^3) in flat-

bottom microtest plates. Target cells were also combined with 0.1 ml of medium alone for determination of spontaneous release of ^{51}Cr . All cultures on the microtest plate were prepared in triplicate. The plates were covered with sterile plastic lids and placed on a rocker platform at 37°C in an atmosphere of 5% CO_2 . The cell suspensions were harvested (with the apparatus described above) after incubation for 24 h. The total amount of ^{51}Cr available for release was determined by placing 0.1 ml of the target cell suspension directly in a gamma counting tube; zero-time samples were also harvested when incubation of the assay materials was begun. To determine lymphocyte-mediated cytotoxicity specific for HSV-1, the amount of ^{51}Cr released spontaneously from infected target cells (I) and control cells (C) was subtracted from the amount released during the interaction of lymphocytes with infected target cells (I') or control cells (C'), respectively. The amount released at zero time was subtracted from the total amount of ^{51}Cr that was available for release during the incubation period. Finally, the specific immune release (SIR) was calculated by subtraction of the percentage of ^{51}Cr released from control target cells from the percentage released from infected cells, according to the following formula: $\% \text{ SIR} = [(I' - I)/(T_i - \text{zero-time}) - (C' - C)/(T_c - \text{zero-time})] \times 100$.

LIF production. LIF was determined by methods previously described (3). Briefly, 30 ml of heparinized blood was obtained from each subject and dextran sedimented to isolate leukocytes. Cells were reconstituted with 10% horse serum to a concentration of 10^8 per ml. Antigens in this assay were herpes group-infected MA-160 cells; a control antigen preparation was uninfected MA-160 cells. The ratio of infected MA-160 cells to leukocytes for these assays was 1:3. A total of 10^6 cells were placed into a test tube, and 50 μl of leukocyte suspension was added at 37°C for 30 min in 5% CO_2 . An agarose medium containing 1% agarose, single-strength tissue culture medium 199, 10% horse serum, 66 IU of penicillin per ml, and 66 μg of streptomycin per ml was transferred to disposable plastic petri dishes (48 by 8.5 mm; Millipore Corp.). Six wells were punched in the gel with a 2.5-mm (external diameter) gel puncher. Agarose plates with the leukocyte antigen mixtures were then incubated at 37°C in a 5% CO_2 -air mixture for 16 to 20 h. At the end of this time, the area of migration of leukocytes was measured, and the percent migration was calculated by dividing the area of migration with antigen by the area of the migration in the control wells, as follows: Percent inhibition = $1.0 - (\text{area of migration for leukocytes with antigen} / \text{area of migration for leukocytes without antigen}) \times 100\%$.

Antiviral agents. The following antiviral agents were evaluated: (i) acyclovir (Burroughs Wellcome Co., Research Triangle Park, N.C.); (ii) adenine arabinoside (ara-A; Parke, Davis and Co., Ann Arbor, Mich.); (iii) cytosine arabinoside (ara-C; The Upjohn Co., Kalamazoo, Mich.); and (iv) idoxuridine (IDU; Calbiochem, San Diego, Calif.). Concentrations of the antiviral agents known to suppress herpes group viruses were employed as follows: ara-A, 3 $\mu\text{g}/\text{ml}$; ara-C, 10 $\mu\text{g}/\text{ml}$; and IDU, 1.5 $\mu\text{g}/\text{ml}$. Although the 50%

effective dose of acyclovir is as low as 0.02 $\mu\text{g/ml}$ against HSV-1, various concentrations up to 20 $\mu\text{g/ml}$ are presented, since no cytotoxicity was observed up to this level.

RESULTS

Mitogen blastogenesis. The effects of the four antiviral agents on mitogen-induced blastogenesis are presented in Fig. 1 to 3. Experiments were repeated five times with lymphocytes from each of 10 adult donors, and results were averaged for these three figures. Ara-C and IDU significantly depressed lymphocyte blastogenic responses to phytohemagglutinin, pokeweed mitogen, and concanavalin A, whereas acyclovir and ara-A demonstrated no significant toxic effects.

These assays were also conducted with variable incubation times ranging from 1 to 7 days, and results were similar to those for the standard

72-h incubation and 5-h $[^3\text{H}]$ thymidine pulse. Inhibition of blastogenic reactivity was not observed until acyclovir concentrations of 50 $\mu\text{g/ml}$ were added, with marked depression noted at 200 $\mu\text{g/ml}$ (Table 1).

Antigen-induced blastogenesis. Table 2 summarizes lymphocyte responses to a fungal and bacterial antigen as well as reactivity specific to herpes group viruses in the presence of antiviral agents. Ara-C severely depressed blastogenesis with all antigens, IDU had an intermediate effect, and acyclovir and ara-A showed no depressant effects on the assays. Results were similar for HSV-1, HSV-2, varicella-zoster virus, and cytomegalovirus.

Lymphocyte cytotoxicity. Cytotoxic responses of HSV-1-, HSV-2-, varicella-zoster virus-, and cytomegalovirus-infected MA-160 cells to normal donor lymphocytes were lower when

FIG. 1. *In vitro* lymphocyte blastogenesis after incubation with phytohemagglutinin, expressed as BI: counts per minute of $[^3\text{H}]$ thymidine uptake for lymphocytes incubated with phytohemagglutinin, divided by uptake after incubation with medium alone. (—) control; (---) acyclovir, 20 $\mu\text{g/ml}$; (****) ara-A, 3 $\mu\text{g/ml}$; (●●●) ara-C, 10 $\mu\text{g/ml}$; (---) IDU, 1.5 $\mu\text{g/ml}$.

FIG. 2. *In vitro* lymphocyte blastogenesis after incubation with pokeweed mitogen expressed as BI: counts per minute of $[^3\text{H}]$ thymidine uptake for lymphocytes incubated with pokeweed mitogen, divided by uptake after incubation with medium alone. Symbols as in Fig. 1.

FIG. 3. *In vitro* lymphocyte blastogenesis after incubation with concanavalin A expressed as BI: counts per minute of [3 H]thymidine uptake for lymphocytes incubated with concanavalin A, divided by uptake after incubation with medium alone. Symbols as in Fig. 1.

TABLE 1. Mitogen-induced blastogenesis in the presence of varying concentrations of acyclovir

Acyclovir concn (μ g/ ml)	% of control BI ^a		
	PHA (5 μ g/ml)	Con A (10 μ g/ml)	PWM (1 μ g/ml)
200	39	27	49
100	66	39	70
50	93	74	89
20	100	100	100
16	100	100	100
12	100	100	100
10	100	100	100
8	100	100	100
4	100	100	100
2	100	100	100
0.4	100	100	100
Control BI ^b	46	31	30

^a Each figure represents the average of five separate experiments, with each experiment done in triplicate. PHA, Phytohemagglutinin; Con A, concanavalin A; PWM, pokeweed mitogen.

^b Counts per minute of [3 H]thymidine uptake for lymphocytes incubated with mitogen, divided by uptake after incubation with medium alone.

ara-C or IDU was included in the tissue culture medium (Table 3). In contrast to these agents, neither acyclovir nor ara-A suppressed release of ^{51}Cr or, presumably, cytotoxicity. However, changes with ara-C and IDU might be in part attributable to toxic effects on the cell lines themselves with resulting increased nonspecific release of ^{51}Cr seen in uninfected control cultures. This increased background tends to magnify what might be small differences.

LIF production. As seen in Table 4, activity of LIF was not significantly altered by any of the antiviral agents studied.

DISCUSSION

Advances in the control of viral disease have

so far resulted primarily from the development of safe and effective vaccines. However, infections following reactivation of latent viruses, such as with members of the herpes group, are not currently amenable to control with active immunization, nor are they completely responsive to therapy with immune globulin or specific antibody-containing plasma. Therefore, attempted therapy of herpesvirus infections has, once again, focused on a chemotherapeutic approach. The greatest limiting factor for the use of these agents has been toxicity resulting from nonspecificity of anti-deoxyribonucleic acid activity. Such toxicity has resulted in the elimination of ara-C (19) and IDU (2) from the systemic antiviral chemotherapeutic armamentarium. Although parenterally administered ara-A has been successful against a limited group of herpesvirus infections with relatively low toxicity (4, 13), safer and more effective compounds are needed. Bioactivation of acyclovir by virus-specified thymidine kinase offers a potential nontoxic candidate for human use (4).

Examination of the immune system is not routinely included in preclinical toxicity testing of investigational drugs because there is a relative lack of standardized guidelines for preclinical screening of antiviral compounds at this time. There is also an incomplete understanding of the contribution of immune mechanisms in combating these diseases. It is generally accepted that recovery from viral disease is directly proportionate to the degree of activity of host immune mechanisms (15). It is therefore important that antiviral chemotherapeutic agents not render the host's immunological responses less effective. This was first appreciated clinically with the therapeutic failures of ara-C (19) and IDU (2), attributed to bone marrow suppression and impaired granulocyte function, but perhaps also a result of immunosuppression by these agents.

TABLE 2. *Specific antigen-induced blastogenesis in the presence of antiviral agents*

Antigen	Control BI ^a	% of control BI			
		Acyclovir (20 μ g/ml)	Ara-A (3 μ g/ml)	Ara-C (10 μ g/ml)	IDU (1.5 μ g/ml)
<i>C. albicans</i>	16.3	100	95	17	61
Tetanus toxoid	13.7	100	88	5	57
Herpes group viruses					
HSV-1	11.4	100	100	0	41
HSV-2	17.7	100	100	0	33
Varicella-zoster virus	8.5	98	100	21	56
Cytomegalovirus	6.8	100	91	4	40

^a Expressed as counts of [³H]thymidine uptake per minute for lymphocytes incubated with antigen, divided by uptake after incubation in medium alone.

TABLE 3. *Lymphocyte cytotoxicity to herpes group-infected target cells in the presence of antiviral agents*

Cells infected with:	Control SIR ^a	% of control SIR			
		Acyclovir (20 μ g/ml)	Ara-A (3 μ g/ml)	Ara-C (10 μ g/ml)	IDU (1.5 μ g/ml)
HSV-1	24.5	100	81	27	64
HSV-2	13.1	100	100	19	71
Varicella-zoster virus	9.9	100	100	52	39
Cytomegalovirus	17.4	100	95	53	20

^a Expressed as percent specific immune release (SIR) of ⁵¹Cr attributable to intracellular virus.

TABLE 4. *Herpes group virus-induced LIF production in the presence of antiviral agents*

Cells infected with:	Control MI ^a (%)	% of control MI			
		Acyclovir (20 μ g/ml)	Ara-A (3 μ g/ml)	Ara-C (10 μ g/ml)	IDU (1.5 μ g/ml)
HSV-1	31	100	100	95	100
HSV-2	19	100	100	100	95
Varicella-zoster virus	26	100	100	96	100
Cytomegalovirus	22	100	100	88	96

^a MI, Migration inhibition. $MI = 1 - (\text{migration of cells with viral antigen} / \text{migration of cells without viral antigen}) \times 100\%$.

The development of simpler assays of cellular immune function now allows direct measurement of immunotoxicity of antiviral agents. Such studies were previously employed to evaluate ara-A and demonstrated that it did not impair host defense mechanisms in vitro (13) nor in vivo (15). The present study has focused on acyclovir, a recently introduced investigational compound, and has expanded the in vitro evaluation of ara-A, ara-C, and IDU. Studies in mice (G. R. Elion, unpublished data) have shown that acyclovir at doses of 100 mg/kg per day does not suppress delayed hypersensitivity, graft-versus-host reaction, complement-dependent cellular cytotoxicity, complement-independent cellular cytotoxicity, or circulating antibody formation to sheep erythrocytes.

Significant toxicity for ara-C and IDU was demonstrated in assays of mitogen- and antigen-induced blastogenesis and lymphocyte-mediated cytotoxicity. However, production of the lymphokine, LIF, was not depressed. This is not

surprising, since LIF production requires protein synthesis which should not be inhibited by agents whose major effect is on deoxyribonucleic acid replication. Lymphocyte cytotoxic responses were also suppressed to about half of control values. Since these responses are not dependent on deoxyribonucleic acid synthesis, such effects are not as readily explained. However, it was apparent that ara-C and IDU had direct toxic effects on the virus-infected cell lines, thus making it more difficult to delineate ⁵¹Cr release attributable to specific lymphocyte responses.

In contrast to ara-C and IDU, neither acyclovir nor ara-A impaired selected cell-mediated responses at concentrations of 20 μ g/ml and 3 μ g/ml, respectively. Since acyclovir actually inhibits some herpes group viruses at concentrations as low as 0.02 μ g/ml (4), at 20 μ g/ml a very high potential therapeutic index is suggested. Inhibition of blastogenic reactivity was not observed until acyclovir concentrations of 50 μ g/ml

ml were added, with marked depression noted at 200 μ g/ml. Even at 200 μ g/ml there was no evidence of cytotoxicity or influence on LIF assays.

In summary, acyclovir appears at least equally as non-immunotoxic as ara-A with respect to many cellular immune mechanisms. These may be important preliminary data for consideration during clinical evaluation of human toxicity and efficacy. As with all new antiviral agents, therapeutic potential for acyclovir must be determined in appropriately designed double-blind clinical trials.

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