

In Vitro Susceptibility of Varicella-Zoster Virus to Acyclovir

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The in vitro susceptibility of five strains of varicella-zoster virus to acyclovir was examined by the plaque-reduction method in human diploid lung cells. The 50% effective doses of acyclovir ranged from 2.06 μM to 6.28 μM in a 7-day assay, with a mean of 3.65 μM . Irreversible inhibition of plaque formation was achieved by drug doses exceeding the 50% effective dose for plaque reduction but nontoxic to the cells. Studies on the relative in vitro susceptibility of varicella-zoster virus and herpes simplex virus types 1 and 2 to acyclovir suggested that varicella-zoster virus is two- to eightfold less susceptible to the drug. The antiviral potency of acyclovir for varicella-zoster virus in vitro was compared with that of several other nucleoside analogs. Analysis of the metabolism of acyclovir in varicella-zoster virus-infected WI-38 cells revealed that, as with herpes simplex virus types 1 and 2, the formation of the triphosphate forms of the drug is specific to viral infection.

Varicella-zoster virus (VZV) is a member of the herpes group which shares several biological features with the well-characterized herpes simplex virus (HSV). These include the ability to establish latency during the primary infection, and subsequently to reactivate despite the presence of circulating antibody (1, 17). Primary varicella infection (chicken pox) in normal non-immune individuals is generally a self-resolving, uncomplicated disease. Natural resolution of either the primary infection or reactivated virus infection (herpes zoster) is more difficult, however, in immunocompromised hosts (19, 26). There is a need for antiviral therapy which would ameliorate the clinical course of infection in this patient population and prevent the systemic complications resulting from disseminated disease.

Cytosine arabinoside and idoxuridine have been found unsatisfactory for the systemic treatment of varicella zoster in these patients due to toxicity (3, 8, 25). Controlled studies with adenine arabinoside (ara-A) have indicated potential usefulness in the treatment of both localized and disseminated disease (16, 27). Due to low solubility of the drug and its deamination in vivo, systemic administration of ara-A requires continuous intravenous infusion of the hospitalized patient.

The nucleoside analog 9-(2-hydroxyethoxymethyl)guanine (acyclovir, previously referred to as acycloguanosine) has been shown to be an effective and specific antiviral agent against HSV types 1 and 2 both in vitro and in vivo (2, 4, 6, 7, 11, 12, 18, 22-24). This antiherpetic agent has demonstrated low toxicity, good metabolic stability, and broad tissue distribution in preclin-

ical trials (9, 24). Therefore, acyclovir can be considered an antiviral agent with great therapeutic potential in humans.

This report describes the in vitro susceptibility of VZV to acyclovir, and compares this sensitivity with that of HSV type 1 (HSV-1) and 2. The antiviral potency of acyclovir against VZV in vitro is compared with that of the antiherpetic compounds idoxuridine, cytosine arabinoside, and ara-A. Finally, preliminary studies of the metabolism of acyclovir in VZV-infected cells are presented.

(A preliminary report of these data was presented at the Proceedings of the 19th Interscience Conference on Antimicrobial Agents and Chemotherapy, 1979.)

MATERIALS AND METHODS

Cells. Human embryonic kidney (HEK Flow 4000, Flow Laboratories, Inc., Rockville, Md.) and human diploid fibroblast cells (WI-38, American Type Culture Collection, Rockville, Md.) were grown in Eagle minimal essential medium supplemented with 10% fetal calf serum (Sterile Systems, Inc., Salt Lake City, Utah) and antibiotics (50 U of penicillin plus 50 μg of streptomycin per ml). HEK cells were used at passage levels of 15 to 17, and WI-38 cells were used at passage levels of 22 to 26.

Virus and plaque-reduction assays. The origin, source and in vitro passage level of the varicella zoster strains used in this study are presented in Table 1.

Virus stocks to be used for cell-associated infections were prepared by mixing infected and uninfected cells at a ratio of 1 to 10. Cultures were trypsinized after 24 to 48 h, and the appropriate number of infected cells was seeded onto confluent cell monolayers. Plaque-reduction assays were performed in 60-mm plastic dishes. The drug was added directly in 0.6% Sea Plaque agarose (Marine Colloids, Inc., Rockland, Maine) over-

lay for 3-day assays (4) or in fluid overlay until the day 4 of long-term incubations, at which time the drug was replenished in an agarose overlay. Cultures were fixed with 10% Formalin in phosphate-buffered saline (7.2 mM Na₂HPO₄, 2.8 mM KH₂PO₄, 0.15 M NaCl), stained with crystal violet, and counted with the aid of a dissecting microscope at 10× magnification. Plaques were measured at 10× magnification using a Bausch and Lomb stereozoom microscope fitted with a micrometer disk.

The number of infectious centers was determined by titration of the trypsinized cell suspension on uninfected confluent monolayers. Cultures were incubated with fluid overlay and observed up to 16 days for plaque formation.

Nucleotide analysis by high-pressure liquid chromatography. Confluent cultures of WI-38 cells were infected with VZV strain Ellen at a 1 to 10 ratio of infected to uninfected cells. At 4 h after infection, the medium was replaced with medium containing ¹⁴C-labeled acyclovir (250 μM; specific activity, 55.6 mCi/mmol), and incubation was continued until 20 h post-infection. Cells were washed with phosphate-buffered saline, harvested, and extracted with perchloric acid. The extracts were analyzed as previously described (11) using a Varian Aerograph LCS-1000 high-pressure liquid chromatograph fitted with a Whatman Partisil PXS 10/25 SAX column.

Compounds. Ara-A was obtained from Parke, Davis & Co. (Detroit, Mich.). Ara-C and 5-iodo-5'-amino-2',5'-dideoxyuridine were purchased from Raylo Chemical Ltd., Alberta, Canada, and 5'-iodo-2'-deoxyuridine was purchased from Schwarz/Mann, Orangeburg, N.Y.

RESULTS

Acyclovir treatment produced an inhibitory effect on VZV replication in WI-38 cells as measured by both plaque size and number in the plaque-reduction assay. A marked difference in plaque size was noted in cultures treated with acyclovir at concentrations below the 50% effective dose (ED₅₀) for reduction in plaque number (see Table 3). The ED₅₀'s for the five VZV isolates tested ranged from 1.25 to 2.5 μM acyclovir (average ED₅₀ was 2.0 μM) in a 3-day plaque-reduction assay. When plaque development was allowed to continue for an additional 4 to 6 days under agarose overlay, an average ED₅₀ value of 3.65 μM was obtained, with a range of 2.06 to 6.28 μM acyclovir (Fig. 1). This shift in ED₅₀ values after extended incubation was prob-

ably due to the slower development of some plaques in the drug-treated cultures.

The effect of acyclovir on plaque progression was then examined, as measured by the actual number of infected cells (Fig. 2). Acyclovir at concentrations of 10 μM or greater suppressed the cell-to-cell spread of VZV strain Ellen. Furthermore, the number of viable infected cells remaining decreased upon continued drug treatment. A similar series of experiments measured the ability of plaque formation to resume after drug removal (Table 2). Results of these studies

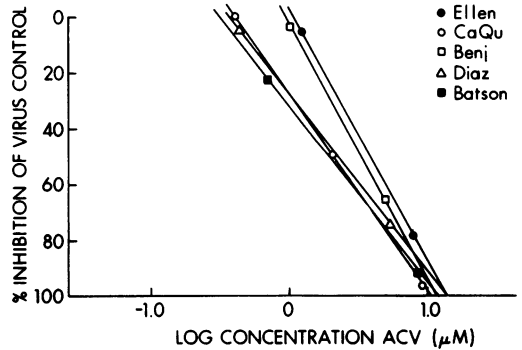


FIG. 1. Plaque-reduction dose-response curves of five strains of VZV to acyclovir in a 7-day plaque-reduction assay in WI-38 cells.

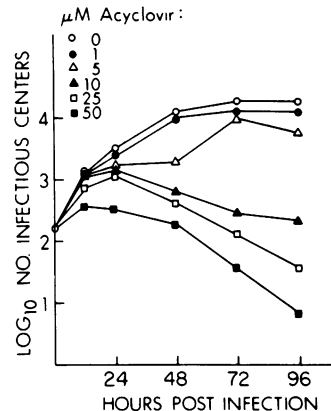


FIG. 2. Effect of acyclovir on cell-to-cell spread of VZV strain Ellen in WI-38 cells, measured by an infectious center assay.

TABLE 1

VZV strain	Origin	Source	Laboratory passage no.
Batson	Herpes zoster (963)	N. J. Schmidt	54
CaQu	Herpes zoster/atypical varicella (1974)	N. J. Schmidt	14
Diaz	Herpes zoster	L. E. Rasmussen	47
Ellen	Varicella	ATCC ^a	8+
Benj	Varicella (1977)	E. S. Huang	6

^a American Type Culture Collection, Rockville, Md.

indicated that cultures infected with VZV strain Ellen could be cured of virus infection by extended treatment with acyclovir at concentrations above the ED₅₀. The length of time required to rid the cultures of infectious virus decreased with increasing drug concentrations. There was a lag time for resumption of plaque formation after drug removal which also correlated with both drug concentration and length of treatment.

The relative susceptibility of VZV and HSV-1 and 2 to acyclovir was examined in WI-38 and HEK cells by the plaque-reduction assay. Time was allowed for maximum plaque development: 3 days in the case of HSV infections, and 7 days for VZV infections. The average ED₅₀ of acyclovir was 0.59 μM for HSV-1 (strains KOS and H29) in WI-38 cells and 0.91 μM for HSV-2 (strains MS and 333). The susceptibility of HSV to inhibition by acyclovir has been shown to vary with host cell species (6, 11); ED₅₀'s of 0.1 to 0.3 μM have been measured for several strains of HSV-1 in Vero cells (11, 13), comparative plaque-reduction assays in HEK cells have yielded average ED₅₀'s of 0.72 μM for strain H29 and 0.74 μM for strain MS, whereas the CaQu and Diaz strains of VZV require drug concentrations of 4.14 and 5.24 μM, respectively (Fig. 3). Results of these studies suggest that VZV is 2- to 8-fold less susceptible to acyclovir than HSV-1 or 2 in these human cells.

The antiviral activity of several antiherpetic compounds was measured against VZV strains Ellen and CaQu in a 7-day plaque-reduction assay (Fig. 4). Cytosine arabinoside and idoxuridine were most active in these assays. The antiviral potencies of ara-A and acyclovir, while slightly lower, were comparable. The addition of an inhibitor of adenosine deaminase did not enhance the activity of ara-A in this cell line (4). The VZV strain Ellen was more resistant to treatment with thymine arabinoside and 5-iodo-5'-amino-2',5'-dideoxyuridine, with mean ED₅₀'s of 36.21 and 117.49 μM, respectively (data not

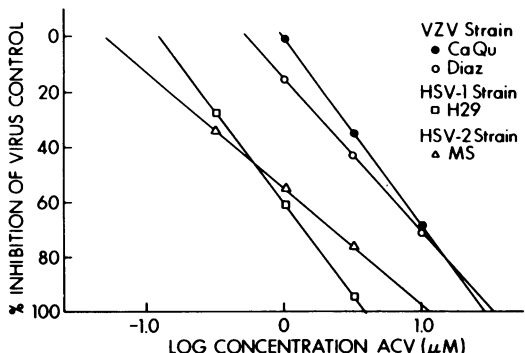


FIG. 3. Inhibition of VZV and HSV-1 and 2 replication in HEK cells by acyclovir in the plaque-reduction assay.

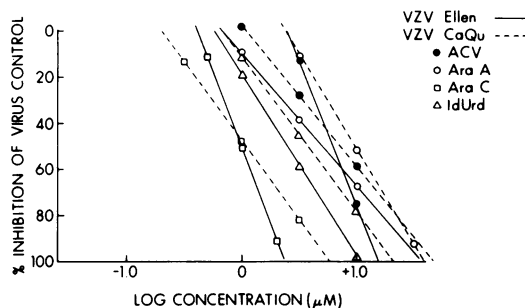


FIG. 4. A comparison of the potency of several nucleoside analogs for VZV strains Ellen and CaQu by the plaque-reduction method in WI-38 cells. ACV, Acyclovir; Ara C, cytosine arabinoside; IdUrd, idoxuridine.

shown).

The antiviral activity of acyclovir has been associated with anabolism of the nucleoside to the triphosphate form in HSV-infected cells (11, 24). VZV-infected WI-38 cells were treated with ¹⁴C-labeled acyclovir for 16 h, harvested, extracted, and analyzed by high-pressure liquid chromatography. Examination of the elution profiles revealed the presence of acyclovir triphosphate in VZV-infected cells. Phosphorylation of the drug occurred specifically in virus-infected cells (Fig. 5). Furthermore, infected WI-38 cells contained higher levels of unconverted nucleoside than did uninfected cells.

DISCUSSION

The in vitro susceptibility of HSV to acyclovir requires the expression of both the viral thymidine kinase and viral deoxyribonucleic acid polymerase (11). The drug is converted to the monophosphate form by the viral thymidine kinase and subsequently to the di- and triphosphate forms by host enzymes (14). The triphosphate form of acyclovir is a competitive inhibitor of the virus-induced deoxyribonucleic acid polymerase (11, 13).

TABLE 2. Extended treatment of VZV-infected WI-38 cells with acyclovir

Concn of acyclovir (μM)	No. of VZV infectious centers after days of acyclovir treatment ^a :					
	0	3	5	7	9	12
10	264.7	215.3	81.6	22.0	3.6	1.0
25	264.7	162.3	19.6	3.0	1.0	0
50	320.0	183.0	62.6	6.0	0	0

^a Values represent average number of viable VZV infectious centers capable of resuming plaque formation after removal of acyclovir on the day indicated. Samples were run in triplicate. VZV strain Ellen was used to infect WI-38 cell cultures in these experiments. Cultures were observed for 14 days after drug removal for plaque formation.

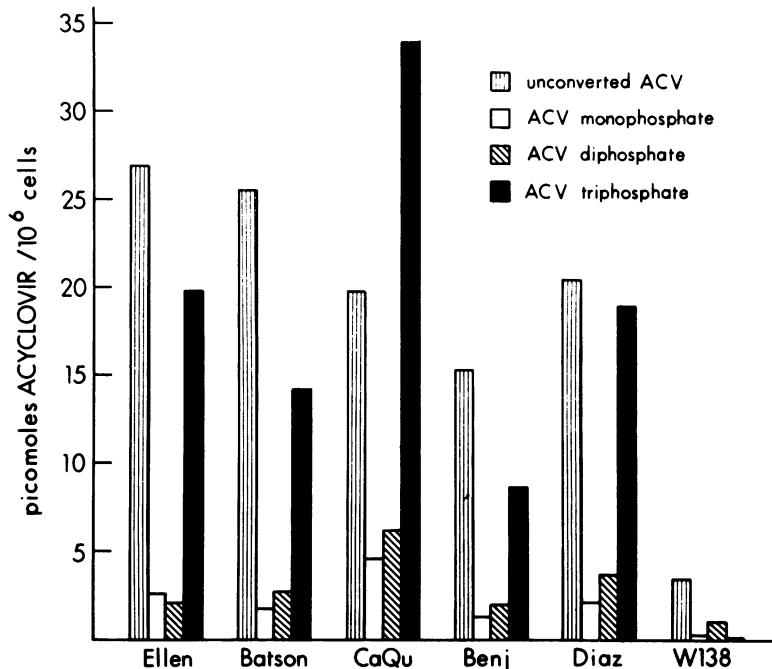


FIG. 5. The metabolism profile of extracts of uninfected human fibroblasts (WI-38) and VZV-infected fibroblasts treated with ¹⁴C-labeled acyclovir (ACV) and analyzed by high-pressure liquid chromatography.

Infection of cells with VZV reportedly induces the synthesis of novel thymidine kinase and deoxyribonucleic acid polymerase activities whose properties differ from those of host cell enzymes (5, 10, 15, 20, 21; J. Fyfe and K. K. Biron, Proceedings of the 19th Interscience Conference on Antimicrobial Agents and Chemotherapy, abstr. no. 783, 1979). The *in vitro* efficacy of acyclovir against clinical isolates of VZV has been reported (7; Y. Bryson, personal communication). Data presented in this report confirm and extend studies of the *in vitro* susceptibility of VZV to acyclovir.

VZV appears to be less susceptible to acyclovir inhibition than HSV-1 or 2 in these human cells, although cell-to-cell spread of the virus is strongly inhibited by drug levels readily achieved in human plasma after parenteral administration (9). Cultures infected with VZV strain Ellen can be cured of infectious virus by treatment with nontoxic concentrations of acyclovir. The cytotoxic effect of acyclovir for growing WI-38 cells was determined by incubating 2×10^5 cells in growth medium containing various drug concentrations. Total cell numbers were counted after 72 h, and 1.0 mM acyclovir, the highest concentration tested, was found to inhibit WI-38 cell growth by 34% (data not shown). No acyclovir triphosphate was detected by high-pressure liquid chromatography in uninfected WI-38 cells. Uptake of the nucleoside appears to be an efficient, selective process in cells infected

by viruses of the herpes group which express a virus-induced thymidine kinase. WI-38 cells infected with human cytomegalovirus, which lacks a detectable virus-induced thymidine kinase, do not accumulate appreciable intracellular levels of the drug (M. St. Clair, unpublished data). Therefore, both the uptake and phosphorylation of acyclovir occur selectively in cells infected by HSV or VZV. The presence of acyclovir triphosphate has been correlated with antiviral activity in HSV-infected cells (11). Studies of the *in vitro* susceptibility of the partially purified VZV deoxyribonucleic acid polymerase to acyclovir triphosphate are in progress.

No significant variation in drug susceptibility was noted in this study based on the isolate source or length of passage in cell culture. The range in ED₅₀ values for any one strain tested in repeated assays was similar to that noted for all five strains when tested simultaneously. Crum-packer et al. reported similar drug susceptibilities for four clinical VZV isolates and observed that cell-free and cell-associated infections were equally susceptible to the drug (7).

The antiviral activity of acyclovir for the CaQu and Ellen strains of VZV compares well with that of ara-A. Measurements of plaque sizes in these experiments demonstrated that at concentrations below the ED₅₀ range, acyclovir was more restrictive on virus spread than was ara-A at comparable concentrations (Table 3).

The demonstrated efficacy of acyclovir for

TABLE 3. Effect of drug treatment on plaque size of VZV strain Ellen

Drug ^b	Plaque size at concn (μM) of drug ^a :						
	0	0.1	0.5	1.0	2.0	3.0	5.0
Acyclovir	2.53	2.28 (0.901)	2.11 (0.833)	1.45 (0.577)	1.24 (0.490)	0.99 (0.391)	0.84 (0.332)
Ara-A	2.54	2.47 (0.970)	2.14 (0.842)	1.94 (0.763)	1.57 (0.618)	1.38 (0.543)	1.29 (0.507)

^a The value for plaque size, expressed in millimeters, represents an average of the measurements of 10 (control cultures) or 20 (drug-treated cultures) plaques per duplicate sample. The numbers within parentheses represent the fractional value of the control plaque size.

^b In this 7-day assay in WI-38 cells, the ED_{50} for acyclovir was 6.24 μM , and the ED_{50} for ara-A was 4.98 μM .

VZV infections *in vitro* suggest that this antiviral agent may be useful in the treatment of patients with varicella-zoster infections.

ACKNOWLEDGMENTS

We gratefully acknowledge the skillful technical assistance of J. E. Noblin and M. H. St. Clair. We thank B. H. Nobles, who prepared the illustrations presented in this report.

LITERATURE CITED

- Bastrain, F. O., A. S. Rabson, C. L. Yee, and T. S. Tralka. 1974. Herpes virus varicella isolated from human dorsal root ganglia. *Arch. Pathol.* **97**:331-333.
- Bauer, D. J., P. Collins, W. E. Tucker, and A. W. Macklin. 1979. Treatment of experimental herpes simplex keratitis with acycloguanosine. *Brit. J. Ophthalmol.* **63**:429-436.
- Boston Interhospital Virus Study Group and NIAID-sponsored Cooperative Antiviral Clinical Study. 1975. Failure of high dose 5-iodo-2'-deoxyuridine in the therapy of herpes simplex virus encephalitis: evidence of unacceptable toxicity. *N. Engl. J. Med.* **292**:599-603.
- Bryson, Y. J., and J. D. Connor. 1976. *In vitro* susceptibility of varicella zoster virus to adenine arabinoside and hypoxanthine arabinoside. *Antimicrob. Agents Chemother.* **9**:540-543.
- Cheng, Y.-C., T. Y. Tsou, T. Hackstadt, and L. P. Mallavia. 1979. Induction of thymidine kinase and DNase in varicella-zoster virus-infected cells and kinetic properties of the virus-induced thymidine kinase. *J. Virol.* **31**:172-177.
- Collins, P., and D. J. Bauer. 1979. The activity *in vitro* against herpes virus of 9-(2-hydroxyethoxymethyl) guanine (acycloguanosine), a new antiviral agent. *J. Antimicrob. Chemother.* **5**:431-436.
- Crumpacker, C. S., L. E. Schnipper, J. A. Zair, and M. J. Levin. 1979. Growth inhibition by acycloguanosine of herpes virus isolated from human infections. *Antimicrob. Agents Chemother.* **15**:642-645.
- Davis, C. M., J. V. VanDersari, and C. A. Coltman, Jr. 1973. Failure of cytarabine in varicella-zoster infections. *J. Am. Med. Assoc.* **224**:122-123.
- De Miranda, P., R. J. Whitley, M. R. Blum, R. E. Keeney, N. Barton, D. M. Cocchetto, S. Good, G. P. Hemstreet, L. E. Kirk, D. A. Page, and G. B. Elion. 1979. Acyclovir kinetics after intravenous infusion. *Clin. Pharmacol. Ther.* **26**:718-728.
- Doberson, M. J., M. Jerkofsky, and S. Greer. 1976. Enzymatic basis for the selective inhibition of varicella zoster virus by 5-halogenated analogues of deoxycytidine. *J. Virol.* **20**:478-486.
- Elion, G. B., P. A. Furman, J. A. Fyfe, P. de Miranda, L. Beauchamp, and H. Schaeffer. 1977. Selectivity of action of an antiherpetic agent, 9-(2-hydroxyethoxymethyl)guanine. *Proc. Natl. Acad. Sci. U.S.A.* **74**:5716-5720.
- Field, H. J., S. E. Bell, G. B. Elion, A. A. Nash, and P. Wildy. 1979. Effect of acycloguanosine treatment on acute and latent herpes simplex infections in mice. *Antimicrob. Agents Chemother.* **15**:554-561.
- Furman, P. A., M. H. St. Clair, J. A. Fyfe, J. L. Rideout, P. M. Keller, and G. B. Elion. 1979. Inhibition of herpes simplex virus-induced DNA polymerase activity and viral DNA replication by 9-(2-hydroxyethoxymethyl)-guanine and its triphosphate. *J. Virol.* **32**:72-77.
- Fyfe, J. A., P. M. Keller, P. A. Furman, R. L. Miller, and G. B. Elion. 1978. Thymidine kinase from herpes simplex virus phosphorylates the new antiviral compound, 9-(2-hydroxyethoxymethyl)guanine. *J. Biol. Chem.* **253**:8721-8727.
- Hackstadt, T., and L. P. Mallavia. 1978. Deoxypyrimidine nucleoside metabolism in varicella-zoster virus-infected cells. *J. Virol.* **25**:510-517.
- Johnson, M. T., J. P. Luby, R. A. Buchanan, and D. Mikulec. 1975. Treatment of varicella-zoster virus infections with adenine arabinoside. *J. Infect. Dis.* **131**:225-229.
- Joncas, J. H. 1979. Persistence, reactivation, and cell transformation by human herpes viruses: herpes simplex 1, 2, cytomegalovirus, varicellazoster, Epstein-Barr virus. *Can. J. Microbiol.* **25**:254-260.
- Kaufman, H. E., E. D. Varnell, Y. M. Centifanto, and S. D. Rheinstrom. 1978. Effect of 9-(2-hydroxyethoxymethyl)guanine on herpesvirus-induced keratitis and iritis in rabbits: *Antimicrob. Agents Chemother.* **14**:842-845.
- Levin, M. J. and J. A. Zaia. 1977. Immunosuppression and infection-progress? *New Engl. J. Med.* **296**:1406-1408.
- Mar, E.-C., Y.-S. Huang, and E.-S. Huang. 1978. Purification and characterization of varicella-zoster virus-induced DNA polymerase. *J. Virol.* **26**:249-256.
- Miller, R. L. and F. Rapp. 1977. Varicella-zoster virus-induced DNA polymerase. *J. Gen. Virol.* **36**:515-524.
- Oxford, J. S. 1979. Inhibition of herpes virus by a new compound—acyclic guanosine. *J. Antimicrob. Chemother.* **5**:333-334.
- Park, N. H., D. Pavan-Langston, S. L. McLean, and D. M. Albert. 1979. Therapy of experimental herpes simplex encephalitis with aciclovir in mice. *Antimicrob. Agents Chemother.* **15**:775-779.
- Schaeffer, H. J., L. Beauchamp, P. de Miranda, G. B. Elion, D. J. Bauer, and P. Collins. 1978. 9-(2-hydroxyethoxymethyl)guanine activity against viruses of the herpes group. *Nature (London)* **272**:583-585.
- Schimpff, S. C., C. L. Fortner, W. H. Greene, and P. H. Wiernik. 1974. Cytosine arabinoside for localized herpes zoster in patients with cancer: failure in a controlled trial. *J. Infect. Dis.* **130**:673-676.
- Schimpff, S., A. Serpick, B. Stoler, B. Rumack, H. Mellin, J. M. Joseph, and J. Block. 1972. Varicella-zoster infection in patients with cancer. *Ann. Intern. Med.* **76**:241-254.
- Whitley, R. J., L. T. Chien, R. Dolin, G. J. Galasso, and C. A. Alford, Jr. 1976. Adenine arabinoside therapy of herpes zoster in the immunosuppressed. *N. Engl. J. Med.* **294**:1193-1199.