An Inhibitor of Herpesvirus Hominis in Extracts of Cultures of Burkitt's Lymphoma Cells (35557)

ALAN S. RABSON, SANDRA A. TYRRELL, AND FRANCES Y. LEGALLAIS

Laboratory of Pathology, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20014

Epstein and his associates first described growth of lymphoid cells from Burkitt's lymphomas in suspension cultures and found, by electron miscroscopy, that some of the cells in the cultures contained viral particles morphologically typical of members of the herpesvirus group (1). These findings have been confirmed in a number of laboratories and the viral particles have been found to be a previously undescribed herpesvirus now known as the EB virus (2). The number of cells containing EB virus particles generally decreases progressively in the cultures and in many of the established cell lines few or no viral particles can be found electron microscopically (3). A critical question related to the possible etiologic role of EB virus in Burkitt's lymphoma has been whether all of the tumor cells contain the viral genome, with the cells containing visible particles representing the lytic cell–virus interaction while the cells without detectable particles are those with the viral genome in a repressed state. In support of the hypothesis that the EB viral genome can exist in a repressed state in lymphoma cells, viral DNA has been demonstrated by nucleic acid hybridization techniques in cell lines free of morphologically detectable virions (4), and soluble antigens which may be EB viral gene products have also been demonstrated in such cell lines (5). Since lysogenic bacteria contain phage repressors (6), and there have been reports of viral inhibitors in extracts of mammalian cells transformed by polyoma virus and simian virus 40 (7, 8), we thought that it would be of interest to search for a herpesvirus inhibitor in Burkitt's lymphoma cells. Because quantitative plaque infectivity titration procedures are not available for EB virus, we have used Herpesvirus hominis (HSV) in our infectivity assays as it seemed possible that an inhibitor of EB virus might also be active against a related herpesvirus.

Materials and Methods. Cells and media. The Burkitt's lymphoma cell line used in these studies was AL-1 derived from a jaw tumor of a Nigerian boy (3). This line contains no EB particles by electron microscopy. In HSV experiments, the following cells were used: a cell line derived from LBN rat kidney (9); secondary cultures of newborn Sprague-Dawley (SD) rat kidneys prepared by trypsinization; primary human embryo kidney cells, and the Vero line of African green monkey kidney cells. Virus pools of HSV were prepared in HEp-2 human epidermoid carcinoma cells. All cultures were grown and maintained in a medium composed of 20% fetal bovine serum and 80% RPMI 1640.

Virus. An oral strain of HSV (strain 11124) was used in all studies. The virus pool was prepared in roller bottles of HEp-2 cells and titered $10^{7.07}$ pfu/ml in a plaque assay with a methyl cellulose overlay on the LBN kidney cells as previously described (10). A pool of vesicular stomatitis virus (Indiana strain) grown in primary chick embryo cultures and containing $10^{8.9}$ pfu/ml was also used.

Extract. AL-1 cultures containing 10^6 cells/ml were concentrated 100× by low speed centrifugation (150*g*) for 10 min to contain 10^8 cells/ml and frozen at —20°. When suitable volumes of this concentrate (10^8 cells/ml) had been collected, they were thawed and 10-ml samples were placed in a 50-ml beaker and sonicated at maximum amperage with a Branson probe sonicator for 1 min. The sonicated material was then centrifuged in a Beckman Model L ultracentrifuge with a No. 40 head for 2 hr at 30,000 rpm

and the supernatant was frozen and stored in liquid nitrogen. A similar extract was prepared from HEp-2 cells (10^8 cells/ml).

Poly-L-lysine. Poly-L-lysine (type 1, Sigma) was prepared in a stock solution containing 1000 μg/ml in serum-free RPMI 1640 and used in the inhibitor experiments at a final concentration of 10 μg/ml to increase cellular uptake of proteins as described by Cassingena and Tournier (7).

Experiments and Results. Two types of experiments were used to demonstrate inhibitory activity of AL-1 extracts against HSV. In one type, the extract was used to inhibit plaque formation on infected monolayers, while in the other type the inhibition of viral growth during a 24-hr period was studied. In both types of experiments, the monolayers were exposed to the extract in the presence of poly-L-lysine for 1 hr prior to infection. The extract was then removed during viral adsorption and added to the cultures again after adsorption.

The plaque inhibition experiments were carried out in the following manner with confluent monolayer cultures of LBN rat kidney cells or primary human embryo kidney cells. The growth medium was removed from the cultures and 0.2 ml of a 1:2 dilution of the extract in medium containing poly-L-lysine (final concentration of poly-L-lysine 10 μg/ml) was added. Control flasks received a 1:2 dilution of growth medium in poly-L-lysine medium. The flasks were incubated at 35° for 1 hr and then washed 3× with growth medium. They were then infected with appropriate dilutions of HSV in 0.2 ml and the virus was adsorbed for 1 hr at 35°. Then 0.2 ml of the 1:2 dilution of extract in poly-L-lysine medium or the control medium were added to each flask and they were incubated for 30 min. Then 6 ml of the methylcellulose overlay containing 20% fetal bovine serum were added to each flask and they were incubated at 35° for 3 days. They were then fixed and stained with methylene blue as previously described (10).

The results of a number of representative experiments are shown in Table I. In all of the experiments there was more than a 3-fold inhibition of HSV plaques in the treated cultures and in some experiments the inhibition was greater than 100-fold. The inhibition was demonstrable both with LBN rat kidney cells and human embryo kidney cells. In contrast to the inhibition of HSV plaques, in similar experiments with Indiana strain of vesicular stomatitis virus (VSV), no significant difference between treated and control cultures was seen.

TABLE I. Plaque Reduction by AL-1 Extracts.

Virus–cell system	No. of plaques in: Extract-treated cultures[a]	Control cultures
HSV[b]-LBN rat kidney	0	100
	6	74
	0	310
	0	88
VSV[c]-LBN rat kidney	38	55
	29	26
	160	148
HSV-human embryo kidney	8	40
	1	10
	6	76
VSV-human embryo kidney	107	142

[a] Mean of 2 replicate 25-cm² Falcon flask cultures.

[b] Herpesvirus hominis.

[c] Vesicular stomatitis virus.

In the 24-hr growth inhibition experiments, medium was removed from flasks containing confluent monolayers and 0.2 ml of a 1:2 dilution of extract in poly-L-lysine medium or control medium with poly-L-lysine were added as in the plaque inhibition experiments. After 1-hr incubation at 35°, the cultures were washed 3 times with 3 ml of growth medium and the flasks were inoculated with 0.2 ml of 10^{-4} dilution of the HSV pool. After 1 hr of adsorption at 35°, 0.2 ml of the 1:2 dilution of extract or control medium with poly-L-lysine were added and the flasks were incubated at 35° for an additional 30 min. Then 5 ml of growth medium were added to each flask. The cultures were incubated for 24 hr and then frozen and thawed. The fluid and cell mixtures were titered by plaque assay for HSV on monolayers of Vero cells. The results of typical experiments are

TABLE II. Viral Growth Inhibition by AL-1 Extracts.

Virus–cell system	Amount (pfu[a]/0.2) ml of virus in: Extract-treated cultures[b]	Control cultures[b]
HSV[c]-LBN rat kidney	$10^{1.6}$	$10^{4.2}$
	$10^{3.2}$	$10^{4.2}$
	$10^{3.5}$	$10^{5.3}$
	$10^{3.4}$	$10^{5.2}$
HSV-SD rat kidney	$10^{1.6}$	$10^{3.8}$
	$10^{2.8}$	$10^{3.8}$
	$10^{2.5}$	$10^{4.4}$
VSV[d]-SD rat kidney	$10^{6.2}$	$10^{8.6}$

[a] Plaque-forming units.

[b] 24 hr after infection; mean of 2 replicate 25-cm² Falcon flask cultures.

[c] Herpesvirus hominis.

[d] Vesicular stomatitis virus.

shown in Table II. The titer of HSV in the control flasks was between 10- and 100-fold greater than that in the flasks treated with the AL-1 extract. In contrast to the marked inhibition of HSV growth, VSV growth was only slightly decreased by treatment with the AL-1 extract. When extracts prepared from HEp-2 cells were used instead of the AL-1 extracts, no inhibition of growth of HSV in secondary cultures of Sprague-Dawley rat kidney cells was observed. Unfortunately, at the time the HEp-2 extracts were available for testing, the line of LBN rat kidney cells had a marked decline in growth rate with degenerative changes in many of the cells. Since secondary Sprague-Dawley rat kidney cells were also sensitive to the inhibitor in the AL-1 extracts, they were used in all subsequent experiments.

Since AL-1 cells produce immunoglobulins *in vitro* (11), the possibility was considered that the extracts contained an HSV neutralizing antibody. To test this, the extracts were used in plaque neutralization tests in which equal amounts of undiluted extract and a 10^{-5} dilution of the virus pool were mixed and incubated at room temperature for 1 hr. As controls, the same amounts of virus were incubated with growth medium. The amounts of virus in the mixtures were assayed and there was no evidence of neutralization of HSV by the extracts.

To determine whether the inhibitor in the extracts was a protein, preparations were tested for HSV inhibitory activity after incubation with trypsin. No decrease in activity was observed after incubation with 125 μg/ml of trypsin but there was a fourfold decrease in activity after incubation with 2 mg/ml of trypsin for 1 hr at 37°.

All of the experiments shown in Tables I and II were done with the extracts diluted 1:2. When the extracts were diluted 1:10 and 1:100, there was no decrease in plaque formation or virus growth of HSV. In one experiment in which a 1:5 dilution of extract was used, there was a reduction of plaques from 61 in the control flasks to 0 in the treated flasks.

Discussion. Although we have demonstrated an inhibitor of HSV in extracts of Burkitt's lymphoma cell cultures, the nature of the inhibitory material is not clear. Immunoglobulins are produced by the AL-1 cells (μ1), however it seems unlikely that the inhibitor is an HSV antibody since the extract showed no activity in a conventional plaque neutralization test. It has been shown that AL-1 culture fluids contain small amounts of human interferon (3), however it seems unlikely that our inhibitor is interferon since the material is active in rat as well as human cells and is not active against VSV, a virus known to be highly susceptible to interferon. The possibility that the inhibitor in the AL-1 extract is an interferon inducer also seems unlikely since the material is highly active against HSV and has little or no activity against VSV. Our assay system was designed to detect an inhibitor that would inactivate HSV after it entered a cell; however it is possible that materials in the extract might adhere to the cell surface even after washing and interfere with adsorption and penetration. The studies with trypsin inactivation suggest that the inhibitor is a protein; however, further purification of the active material from the concentrated cell extracts is necessary before any real characterization can be undertaken.

If it is true that Burkitt's lymphoma cells

all contain the EB viral genome in some unexpressed or partially expressed state (4, 5), it seems possible that the HSV inhibitor in our A-1 extracts may also be involved in the inhibition of EB virus within the Burkitt's lymphoma cells. Purification of the inhibitor and determination of its mechanism of action should clarify this point.

Summary. An inhibitor of Herpesvirus hominis is present in extracts of a lymphoid cell line derived from a Burkitt's lymphoma. The inhibitor is demonstrable when monolayer cultures of rat and human cells are treated with the extracts, and although it is active against the herpesvirus in both plaque inhibition and 24-hr growth inhibition experiments, it does not significantly inhibit vesicular stomatitis virus in similar experiments. The inhibitor does not appear to be interferon or an interferon inducer, and the possibility that it is involved in the maintenance of the EB viral genome in an unexpressed or partially expressed form in Burkitt's lymphoma cells is considered.

The authors are grateful to Mrs. Myrtle Whiting for technical assistance.

1. Epstein, M. A., and Barr, Y. M., J. Nat. Cancer Inst. **34**, 231 (1965).

2. O'Conor, G. T., Amer. J. Med. **48**, 279 (1970).

3. Rabson, A. S., O'Conor, G. T., Baron, S., Whang, J. J., and Legallais, F. Y., Int. J. Cancer **1**, 89 (1966).

4. Zur Hausen, H., and Schulte-Holthausen, H., Nature (London) **227**, 245 (1970).

5. Vonka, V., Benyesh-Melnick, M., and McCombs, R. M., J. Nat. Cancer Inst. **44**, 865 (1970).

6. Ptashne, M., Proc. Nat. Acad. Sci. U.S.A. **57**, 306 (1967).

7. Cassingena, R., and Tournier, P., C. R. Acad. Sci. **267**, 2251 (1968).

8. Cramer, R., C. R. Acad. Sci. **268**, 3142 (1969).

9. Rabson, A. S., Edgcomb, J. H., Legallais, F. Y., and Tyrrell, S. A., Proc. Soc. Exp. Biol. Med. **131**, 923 (1969).

10. Rabson, A. S., Tyrrell, S. A., and Legallais, F. Y., Proc. Soc. Expt. Biol. Med. **132**, 802 (1969).

11. Fahey, J. L., Finegold, I., Rabson, A. S., and Manaker, R. A., Science **152**, 1259 (1966).

Received Oct. 14, 1970. P.S.E.B.M., 1971, Vol. 137.