Abortive Herpes Simplex Virus Replication in Rous Sarcoma Virus Transformed Cells (37665)

JOHN J. DOCHERTY, WAYNE R. MITCHELL, AND CHARLES J. THOMPSON
(Introduced by A. Anthony)

Department of Microbiology, The Pennsylvania State University, University Park, Pennsylvania 16802

Studies by Van Der Noordaa, Enders, and Diamondopoulos (1) originally demonstrated that herpes simplex virus (HSV) does not replicate normally in SV40 transformed cells. Subsequently, others produced results which indicated that HSV replicated at reduced levels in a Burkitt lymphoma cell culture, EB3 (2). Furthermore, Rabson, Tyrell and Legallais (3) were able to isolate an inhibitor of HSV in extracts of a lymphoid cell line derived from Burkitt's lymphoma.

The above studies establish that in some cells transformed by DNA viruses an alteration has occurred which is deleterious to the replication of HSV. However, no studies have been directed at HSV replication in cells transformed by viruses of the oncornavirus group. Therefore, our studies were designed to determine if a cell transformed by an RNA virus would resist HSV infection similar to cells which were transformed by DNA viruses.

In 1960 the XC cell line was developed from tumor cells induced in newborn Wistar albino rats by the Prague strain of Rous sarcoma virus (4). Most notably this cell line has been used extensively to quantitate murine leukemia viruses *in vitro* (5).

Our attempts to propagate herpes simplex virus type 1 (HSV-1) and herpes simplex virus type 2 (HSV-2) in the XC cell line were unsuccessful. However, both HSV-1 and HSV-2 were able to replicate to high levels in control nontransformed Wistar rat embryo cells. Our studies further demonstrate that both HSV-1 and HSV-2 will attach to XC cells, but produce minimal viral antigens, do not induce viral DNA, do not depress cell DNA synthesis, and do not induce detectable amounts of virus specific thymidine kinase.

Materials and Methods. Cells. The XC cell line was obtained from Dr. C. Whitmire of Microbiological Associates, Inc., Bethesda, MD. These cells were maintained in growth medium consisting of minimal essential medium with Hanks' salts supplemented with 10% newborn calf serum, 20 m*M* L-glutamine, 1 m*M* sodium pyruvate, IX nonessential amino acids (Grand Island Biological, Inc., Grand Island, NY), 100 units/ml penicillin, 10 μg/ml streptomycin, and 0.075% $NaHCO_3$.

The XC cells evolved from Wistar rat cells, therefore Wistar rat embryo cells were selected to serve as normal nontransformed control cells. These were prepared after obtaining 15 day old Wistar rat embryos (WRE) by cesarean section, mincing the embryos with scalpel blades, dispersing the tissue fragments with 0.25% trypsin and seeding resulting cells into 8 oz prescription bottles with growth medium. Within 24 hr these cells had formed monolayers consisting predominantly of fibroblasic-like cells. These cells were passaged after which several bottles were harvested, resuspended in growth medium plus 10% dimethylsulfoxide, and preserved in liquid nitrogen. The WRE cells were maintained in growth medium and used in experiments only until they reached their 20th tissue culture passage. At that time, fresh low passage cells were retrieved from liquid nitrogen and the cycle was repeated.

Virus. All virus stocks (Seibert strain of HSV-1 and 316-D strain of HSV-2) were obtained from Dr. F. Rapp of the Department of Microbiology, M. S. Hershey Medical Center, The Pennsylvania State University, Hershey, PA. Virus stocks were prepared in hu-

man embryonic lung cells and titrations were performed in primary rabbit kidney monolayers utilizing a 0.5% methyl cellulose overlay as previously described (6).

Virus growth studies. Studies were designed to determine if HSV-1 or HSV-2 would replicate in the XC cell line. Cultures of XC cells or WRE cells were dispersed with 0.25% trypsin and placed in 1 oz prescription bottles. As the cells reached confluence (24–48 hr) they were infected with HSV-1 or HSV-2 at a multiplicity of infection (m.o.i) of 3. After 1 hr of adsorption at room temperature with intermittent manual rotation the cells were rinsed once with Tris buffer (0.025 *M* Tris saline, pH 7.4) prior to adding 5 ml of growth medium to each bottle. The 0 hr sample was taken at this time and the remaining samples at 24 hr intervals for 120 hr. These samples were rapidly frozen with dry ice and maintained at —70° until all had been collected. The samples were processed as a group by rapidly thawing, sonicating 10 sec in a 60 W MSE ultrasonic disintegrator (Measuring & Scientific Equipment Ltd., London), and immediately titrated on rabbit kidney monolayers. Results are expressed as plaque forming units per milliliter (pfu/ml).

Virus attachment study. Experiments were designed to determine if HSV-1 and HSV-2 were able to attach to the XC cells. The cells were harvested into growth medium by scraping them off the surface of tissue culture vessels with the aid of a rubber policeman. The cells (10^8) and virus (10^6 pfu) were placed in a final volume of 2 ml of growth medium. Virus stability control consisted of the virus in 2 ml of growth medium. All tubes were incubated at 25° and were agitated at regular intervals. At 0, 10, and 30 min 0.1 ml samples were withdrawn and diluted in 9.9 ml blanks of growth medium to stop virus attachment. These samples were centrifuged at 500*g* for 10 min after which the amount of unattached virus in the supernate fluid was immediately determined by titration in rabbit kidney monolayers.

Thymidine kinase assay. Cultures of XC cells or WRE cells were prepared in 8 oz prescription bottles and infected with HSV-1 or HSV-2 at a m.o.i. of 10. Following 20 hr of infection the cells were rinsed three times with cold (4°) Tris buffer, scraped into 5 ml of cold Tris buffer, and recovered by centrifugation. The supernate was discarded and the cells were resuspended in 1 ml of cold 0.15 *M* KCl in 0.05 *M* Tris buffer (pH 8.0). The cells were then sonicated for 30 sec in a 60 *W* MSE ultrasonic disintegrator prior to centrifuging at 39,100*g*/30 min. The reaction mixture consisted of 0.125 ml of 5 m*M* ATP, 0.05 ml of 5 m*M* $MgCl_2$, and 0.05 ml of [^{3}H] TdR (4 μCi/ml, sp ac 20 Ci/mmole). To the reaction mixture 0.05 ml of cell extract was added after which the samples were incubated at 38° for 15 min. The reaction was terminated by placing the tubes containing the reactants and product in boiling water for 2 min. Coagulated protein was removed by centrifugation and 0.25 μliter spotted on Whatman DE-81 filter paper. These filters were rinsed three times in 1 m*M* ammonium formate, once in distilled water, and once in absolute ethanol.

The filters were dried at 37° for 30 min and oxidized with a Packard Model 300 tritium oxidizer. The resulting tritiated water was collected in a dioxane base scintillation fluid after which the samples were counted with a Packard Model 2002 spectrometer. Protein determinations were made according to the method of Lowry *et al.* (7). Under these conditions, the enzyme activity was linearly related to both the amount of cellular protein and the time of incubation. Specific activity is defined as the nanomoles of [^{3}H] TdR nucleotide produced in the reaction per 15 min per milligram of protein in the cellular extracts.

To determine if the observed enzymatic activity was virus specific, temperature characterization was carried out by holding aliquots of cell extract at 41° for 15 min (8). At the end of this period 0.05 ml of the extract was immediately placed with the reaction mixture and processed as previously described.

Virus protein synthesis. To determine if HSV proteins were synthesized cultures of XC or WRE cells were prepared on sterile 11 × 22 mm coverglasses in 15 × 60 mm tissue culture dishes containing 5 ml of growth medium. Following 24 hr incubation the me-

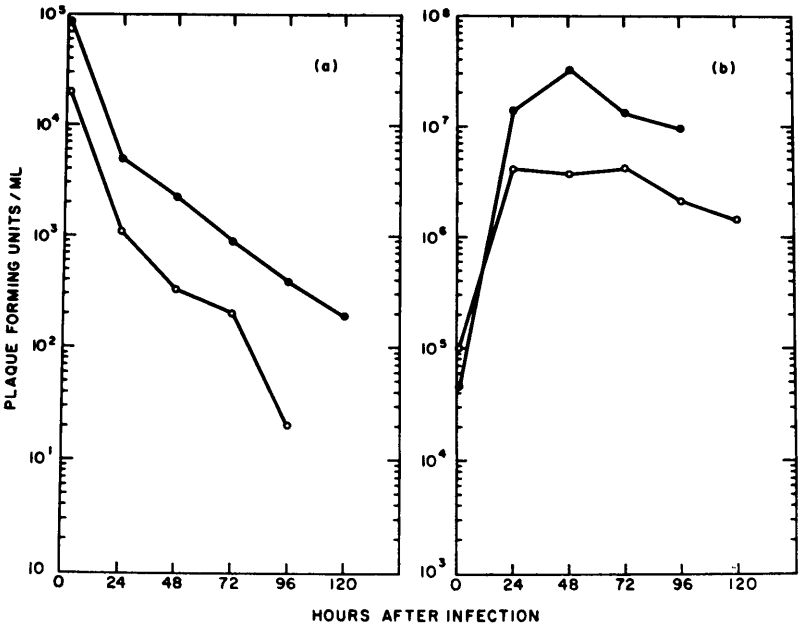

FIG. 1a. The abortive replication of HSV-1 (●-) and HSV-2 (○-) in XC cells. (b) The productive replication of HSV-1 (●-) and HSV-2 (○-) in WRE cells.

dium was removed, the number of cells per coverslip was determined, and then either cell type was infected with HSV-1 or HSV-2 at a m.o.i. of 3. The virus was permitted to adsorb to the cells for 1 hr after which unattached virus was removed by rinsing with Tris buffer and then 5 ml of growth medium was added to all cultures. Representative samples were harvested at 0, 12, 24, 48, and 72 hr after the initial infection. The cells were rinsed three times in Tris buffer (37°), dried at 37° for 20 min, and dehydrated in acetone for 3 min at room temperature. These cells were stored at 4° until all samples were collected and then stained for viral antigens as previously described (9).

Viral and cellular DNA synthesis. The XC or WRE cells were seeded in 1 oz prescription bottles and infected 24–48 hr later with HSV-1 or HSV-2 at a m.o.i. of 3 for 1 hr at room temperature with intermittent manual rotation. At various times after the initial infection [^{3}H]-thymidine (TdR; sp act 20 Ci/mmole; New England Nuclear) was added to representative samples at a final concentration of 5 μCi/ml of growth medium. After 1 or 24 hr of exposure to the isotope the samples were frozen and maintained at −20° until all samples had been collected. A 24 hr sample had isotope added at the time of infection and remained in contact with the infected cells for 24 hr prior to harvest. Likewise, a 48 hr sample had isotope added at 24 hr after infection and remained in contact with infected cells for 24 hr at which time it was harvested as the 48 hr sample and so on. These samples were processed together in order to insure uniform treatment of each specimen before cellular DNA was separated from viral DNA as previously described (9).

Results. Viral replication in XC and WRE cells. A comparative growth study of HSV-1 or HSV-2 in XC or WRE cells is presented in Fig. 1a and b. These results clearly reveal that both HSV types were unable to replicate in the XC cell line but were able to replicate in the control WRE cells.

The amount of HSV-1 or HSV-2 present in the XC cells after the 0 hr determination dropped continuously during the 120 hr the cultures were monitored (Fig. 1a).

Contrasting these results was HSV-1 or HSV-2 infection of WRE cells (Fig. 1b). HSV-1 replicated to its highest level at 48 hr after the primary infection. HSV-2 reached

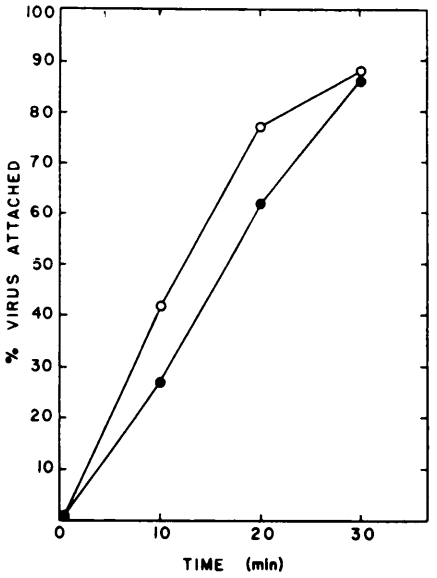

FIG. 2. The attachment kinetics of HSV-1 and HSV-2 to XC cells. (●-) HSV-1 + XC cells, (○-) HSV-2 + XC cells.

its highest level of replication by 24 hr after infection followed by a gradual decrease in the number of infectious particles. These studies were repeated at a m.o.i. of 1, 5, or 10 and the results were the same.

Virus attachment. Since HSV does not replicate in XC cells a possible site of abortive infection could be one of the first obstacles a virus encounters, the attachment of the infectious particle to the plasma membrane. To determine if HSV was able to attach to the XC cells these studies were undertaken and the results are presented in Fig. 2. Both viruses attached rapidly and at similar rates to the XC cells. By 30 min after exposure to cells 86% of HSV-1 and 88% of HSV-2 had attached to the XC cells. Virus instability during this period was negligible.

Viral TdR kinase induction. Recent studies have demonstrated increased levels of TdR kinase in HSV infected cells which appear to be virus specific (10) as well as type specific (11). To determine if the enzyme was present in our nonpermissive system, XC and WRE cells were infected and processed for cell and virus specific TdR kinase as outlined in Methods. Table I presents the results of these studies. Significant levels of TdR kinase were induced in WRE cells infected with HSV-1 or HSV-2. The level of enzyme induction by HSV-1 was 15-fold above uninfected WRE cells while induction by HSV-2 was 7-fold above uninfected WRE cells. Furthermore, these enzymes were specific for each virus type based on heat stability studies. Cell enzyme was most stable at 41° for 15 min, while HSV-1 TdR kinase was less stable, and HSV-2 TdR kinase was least stable of all.

The uninfected XC cells had a higher level of TdR kinase activity than its counterpart, uninfected WRE cells. Furthermore, after infection with either virus type this level did not appreciably increase or decrease and was stable at 41° for 15 min. These studies suggest a lack of virus specific TdR kinase induction in XC cells.

Viral protein synthesis. Certain HSV abortive infections result in the synthesis of viral proteins (12). To determine if viral proteins were synthesized in the abortive XC system these cells were examined by the indirect immunofluorescent technique at various times

TABLE I. TdR Kinase Induction in WRE Cells or XC Cells.

Cell + treatment	TdR kinase activity[a]	Residual TdR kinase activity[b]	% Residual TdR kinase activity
WRE control	0.0478	0.0338	70.7
WRE + HSV-1	0.6900	0.2557	37.1
WRE + HSV-2	0.3224	0.0307	9.5
XC control	0.1669	0.1765	100
XC + HSV-1	0.1604	0.1624	100
XC + HSV-2	0.1668	0.1746	100

[a] Nanomoles [^{3}H]TdR phosphorylated per milligram of protein per 15 min at 38°.
[b] Cell extract placed for 15 min at 41° prior to assaying for TdR kinase activity.

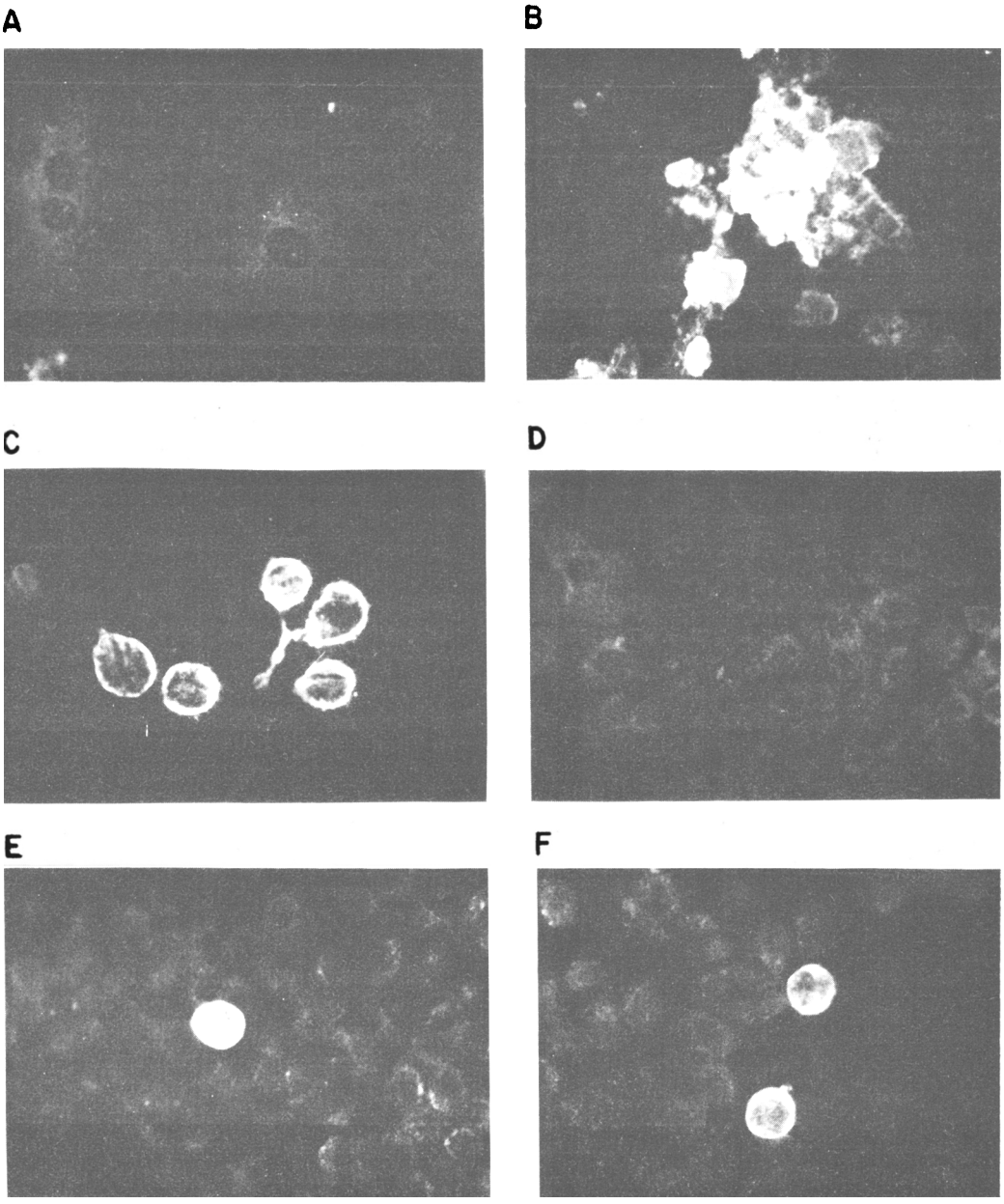

FIG. 3. Detection of HSV specific antigens in WRE cells and XC cells: (A) Noninfected WRE cells, (B) WRE cells + HSV-1, 24 hr postinfection, (C) WRE cells + HSV-2, 24 hr postinfection, (D) XC cells + HSV-1, 24 hr postinfection, (E, F) XC cells + HSV-2, 24 hr postinfection (final magnification 1040×).

after infection.

Coverslip cultures of XC or WRE cells were prepared and infected with HSV-1 or HSV-2 at a m.o.i. of 3 as described in Methods. The WRE cells were extremely receptive to HSV-1 or HSV-2 infection and by 24 hr after exposure to either virus exhibited extensive cell destruction as well as positive virus specific fluorescence (Fig. 3B, C). However, XC cells infected with HSV-1 were to-

tally void of positive fluorescence (Fig. 3D). If the XC cells were infected with HSV-2 an occasional cell would round and react with virus specific immunofluorescent reagents (Fig. 3E, F). The number of positive cells never exceeded 1% during the 72 hr the cultures were monitored. These studies were repeated at m.o.i. of 1 and 5 and the results were the same.

Cell and viral DNA synthesis. The primary objective of these studies was to determine if any virus specific DNA was synthesized in the abortive XC system. Initial studies utilized 1 hr [^{3}H]TdR pulses at various intervals after infection and resulted in the detection of no viral DNA. In order to minimize the possibility that our 1 hr pulses were administered at a time other than active viral DNA synthesis we then exposed our system to [^{3}H]TdR for 24 hr at a time.

Figure 4 presents the results of both WRE cells plus virus and XC cells plus virus after these systems were exposed to isotope for 24 hr periods. Figure 4a presents the DNA profile of noninfected WRE cells, while Fig. 4b and 4c were WRE cells 24 hr after infection with HSV-1 and HSV-2, respectively. In the noninfected WRE cells only one peak is present which corresponds in density to that of mammalian cells (p = 1.700 g/ml). When infected with HSV-1 (Fig. 4b) or HSV-2 (Fig. 4c) there is a reduction in the amount of cellular DNA produced plus the appearance of a second peak which corresponds in density to that of herpes simplex virus (13; p = 1.726–1.728 g/ml).

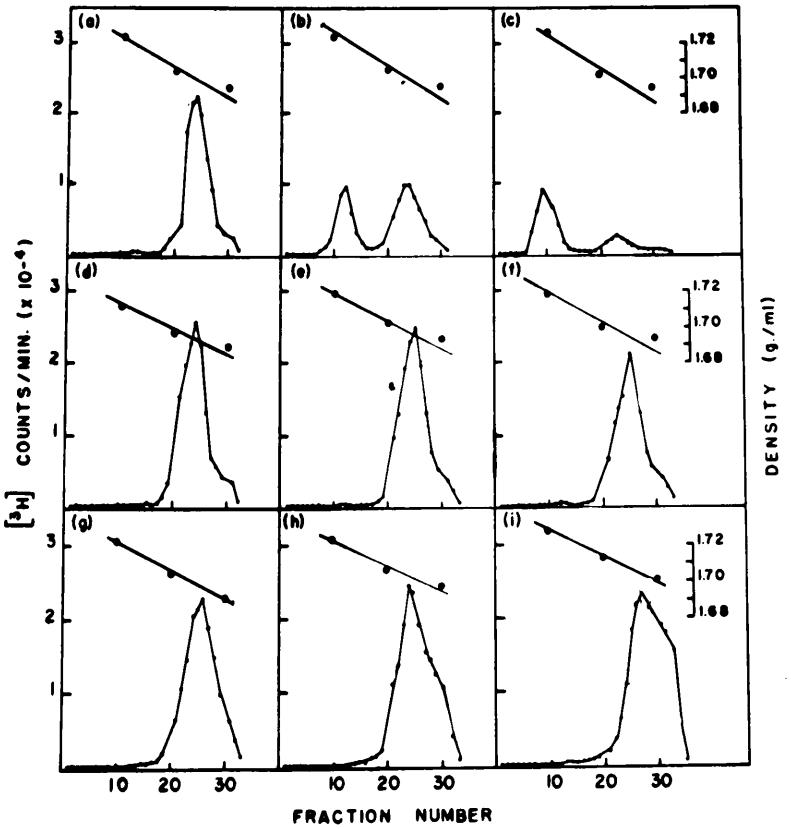

FIG. 4. Detection of cellular or viral DNA in WRE cells or XC cells infected with HSV-1 or HSV-2 and exposed to [^{3}H] thymidine for 24 hr intervals. (a) WRE cells—noninfected, (b) WRE cells + HSV-1, 24 hr postinfection, (c) WRE cells + HSV-2, 24 hr postinfection, (d) XC cells—noninfected 24 hr control, (e) XC cells + HSV-1, 24 hr postinfection, (f) XC cells + HSV-2, 24 hr postinfection, (g) XC cells—noninfected 96 hr control, (h) XC cells + HS-1, 96 hr postinfection, (i) XC cells + HSV-2, 96 hr postinfection.

Figure 4d represents the single cell peak profile of uninfected XC cells. Figure 4e and f are XC cells infected with HSV-1 or HSV-2 for 24 hr. A major peak of cell DNA is observed with virtually no viral DNA present. However, a very small increase in counts was observed between fraction 8 and 12 in Fig. 4f which would correspond to viral DNA. This area represents less than 1% of the counts found in the cellular peak. Figure 4g, h, i presents results of XC control, XC + HSV-1, and XC + HSV-2, 96 hr after infection. The results are similar to the 24 hr XC-virus data except no viral DNA appeared. Furthermore, the decrease in cellular DNA synthesis seen in HSV infected WRE cells did not occur in HSV infected XC cells. XC cells infected with HSV-1 or HSV-2 for 48 or 72 hr presented profiles very similar to those of the 96 hr sample including no increase in the area that would correspond to viral DNA.

Discussion. The results of the present study demonstrate the failure of HSV-1 or HSV-2 to replicate or significantly induce any virus specific alterations of XC cells. Although both viruses were able to replicate to high levels in nontransformed WRE cells and attach to XC cells they were unable to induce viral proteins, DNA, TdR kinase, or depress cell DNA synthesis in the XC cell line.

During the replicative cycle of HSV one of the first barriers or obstacles it must overcome is attachment to the cell. Several reports on the abortive infection of various cells by HSV have been made but none have found the limiting factor to be the inability of the virus to attach to the plasma membrane (1, 9, 14, 15). Our results are similar in that both HSV types were able to attach to the XC cells even though they were unable to replicate in them. Although the virus was able to attach to the XC cells our data does not indicate if HSV is successfully penetrating and uncoating.

After entry early events in HSV replication include the synthesis of a variety of enzymes including TdR kinase (16). However, we were unable to detect TdR kinase in XC cells infected with HSV even at 20 hr after infection when significant levels of enzyme would ordinarily have accumulated. By contrast, both viruses induced significant levels of TdR kinase in nontransformed WRE cells.

Following early enzymes, a second group of major proteins appear in permissive cells which are the structural proteins. Using immunofluorescence it has been demonstrated that in restricted HSV replication due to arginine deprivation these proteins accumulate in the cytoplasm (12). However, our system revealed that these proteins are synthesized in minimal amounts and in a very few cells. These cells were examined from 12 to 72 hr after infection, but no more than 1% of the cells demonstrated positive fluorescence at any one time.

By designating the appearance of viral DNA as the midpoint in the replication of HSV we can arbitrarily divide the replicative cycle in half. As our data establishes, HSV never reaches this midpoint after infection of the XC cells. We were unable to demonstrate viral DNA using various m.o.i. from 1 to 10, various pulses of isotope of 1 and 24 hr, and examination of the system up to 96 hr after infection. The results did not change with various combinations of these parameters.

Furthermore, it is well established that cell DNA synthesis is reduced while viral DNA is synthesized in cells permissive to HSV replication (17, 18). This is amply supported by our results of HSV infection of WRE cells. However, when XC cells were infected with HSV there was no detectable effect on cell DNA synthesis.

The inability of HSV to replicate in certain DNA virus transformed cells has been established (1–3). Our results now indicate that an RNA virus transformed cell is also resistant to HSV replication. Furthermore, it has been demonstrated that viruses of the picorna (19, 20), orthomyxo, and paramyxo (21) group can interfere with the replication of HSV. However, our studies do not indicate if oncornaviruses can directly interfere with HSV, but this possibility is currently being investigated.

Summary. The XC cell line was developed from tumor cells induced in newborn Wistar rats by the Prague strain of Rous sarcoma virus (RSV). Attempts to propagate herpes

simplex virus type 1 (HSV-1) or herpes simplex virus type 2 (HSV-2) in these cells proved futile. However, both virus types were able to replicate to high levels in control nontransformed Wistar rat embryo (WRE) cells.

Examination of this abortive infection revealed that both HSV-1 and HSV-2 were able to attach to the XC cells but were unable to induce virus protein, DNA, or specific thymidine kinase. Furthermore, while cell DNA synthesis was severely depressed in receptive WRE cells, no such effect was observed in resistant XC cells. These studies suggest that an early event, prior to viral DNA synthesis, is blocked in the replicative cycle of HSV.

This study was conducted in part under support funds from the U.S. Public Health Service Biomedical Grant No. 5 S05 R RO7082-07 and the Pennsylvania Agriculture Experiment Station Project No. 1992. Authorized for publication as paper No. 4439 in the journal series of the Pennsylvania Agriculture Experiment Station.

1. Van Der Noordaa, J., Enders, J. F., and Diamandopoulos, G. T., Proc. Soc. Exp. Biol. Med. **122**, 915 (1966).

2. Floyd, R., Glaser, R., Vonka, V., and Benyesh-Melnick, M., Acta Virol. **133**, 133 (1971).

3. Rabson, A. S., Tyrell, S. A., and Legallais, F. Y., Proc. Soc. Exp. Biol. Med. **137**, 264 (1971).

4. Svoboda, J., Nature (London) **186**, 980 (1960).

5. Rowe, W. P., Pugh, W. E., and Hartley, J. W., Virology **42**, 1136 (1970).

6. Docherty, J. J., Goldberg, R. J., and Rapp, F., Proc. Soc. Exp. Biol. Med. **136**, 328 (1971).

7. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., J. Biol. Chem. **193**, 265 (1951).

8. Ogino, T., and Rapp, F., Virology **46**, 953 (1971).

9. Docherty, J. J., Mäntyjärvi, R. A., and Rapp, F., J. Gen. Virol. **16**, 255 (1972).

10. Lowry, S. P., Bresnick, E., and Rawls, W. E., Virology **46**, 958 (1971).

11. Thoules, M. E., J. Gen. Virol. **17**, 307 (1972).

12. Courtney, R. J., McComb, R. M., and Benyesh-Melnick, M., Virology **40**, 379 (1970).

13. Russell, W. C., and Crawford, L. V., Virology **22**, 288 (1964).

14. Aurelian, L., and Roizmăn, B., Virology **22**, 452 (1964).

15. Lowry, S. P., Bronson, P. L., and Rawls, W. E., J. Gen. Virol. **11**, 47 (1971).

16. Ogino, T., Shiman, R., and Rapp, F., Intervirology **1**, 80 (1973).

17. Roizman, B., and Roane, P. R., Virology **22**, 262 (1964).

18. Cohen, G. H., Vaughn, R. K., and Lawrence, W. C., J. Virol. **7**, 783 (1971).

19. Saxton, R. E., and Stevens, J. G., Virology **48**, 207 (1972).

20. Fletcher, R. D., and Jayavasu, C., Arch. Gesamte Virusforsch. **38**, 105 (1972).

21. Szanto, J., and Lesso, J., Acta Virol. **15**, 45 (1971).

Received June 29, 1973. P.S.E.B.M., 1973, Vol. 144.