Proc. Nat. Acad. Sci. USA
Vol. 73, No. 2, pp. 646–650, February 1976
Microbiology

Activation of an endogenous mouse type C virus by ultraviolet-irradiated herpes simplex virus types 1 and 2

(provirus/type C virus activation/herpes simplex virus oncogenesis/herpes simplex virus transformation)

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Communicated by Robert J. Huebner, December 3, 1975

ABSTRACT **Infection of BALB/c mouse cells with UV-irradiated herpes simplex virus (HSV) types 1 and 2 resulted in activation of a xenotropic type C virus detected by infectious center formation in permissive rat cells. The levels of type C virus activated by HSV were related to the UV dose and the multiplicity of infection used. The ability of HSV to activate type C virus was eliminated by heat-inactivation and by neutralization with specific antiserum against HSV, but was not affected by purification or treatment with DNase and RNase. Maximum levels of type C virus in the cells and medium were observed within 1 day after HSV infection, and the levels returned to control cell values within 3–4 days. The possible significance of these findings with respect to the putative oncogenic potential of HSV is discussed.**

Cells of many, if not all, mammalian species contain repressed genomes of type C RNA viruses. Activation of these repressed genomes with resultant virus synthesis may occur spontaneously (1) or may be induced by biological (2), chemical (3–6), or physical (7) means. Evidence exists to suggest that the genomes of these vertically transmitted endogenous RNA viruses can play a role in spontaneous tumor induction (8, 9).

Viruses containing DNA rather than RNA genetic material have also been implicated in the oncogenic process. Inoculation of herpesviruses into animals (10) or cells in culture (11), for example, may be followed by the appearance of tumors or cell transformants that possess malignant potential. The putative role of herpesviruses in the oncogenic process is not clearly understood, however, and one may question whether the herpesvirus genome is in itself oncogenic or whether herpesviruses serve an indirect function by affecting a cellular factor(s) which is the direct oncogen. An example consistent with an indirect role in oncogenesis would be modification of expression of endogenous type C virus information after herpesvirus infection.

Evidence is presented here that herpes simplex virus (HSV) types 1 and 2, inactivated by UV-irradiation, can activate an endogenous mouse type C virus. After the initial observations made in the senior author's laboratory, studies were carried out in collaboration with S.A.A., and the results from both laboratories are presented here.

MATERIALS AND METHODS

Cells and Media. Two murine nonproducer (NP) cell lines derived from BALB/3T3 cells were used. The K-BALB cell line contains rescuable Kirsten murine sarcoma virus information and is oncogenic in BALB/c mice (12). The 43-2 cell obtained from Dr. Masakazu Hatanaka (Flow Laboratories) was isolated from K-BALB cells that had been treated with BrdUrd, and is poorly oncogenic in BALB/c mice (13). Additional cells included the normal rat kidney (NRK) cell line (14), primary BALB/c and NIH Swiss mouse embryo cells, and the Vero cell line. Cells were grown either in minimal essential medium supplemented with 10% heat-inactivated fetal calf serum and antibiotics, or in Dulbecco's modified Eagle's medium supplemented with 10% calf serum and antibiotics.

HSV Strains. The type 1 14-012 strain (obtained from Dr. Fred Rapp, Pennsylvania State), and the type 2 Savage (obtained from Dr. Ann Boyd, Frederick Cancer Research Center), and MS strains of HSV were used. All strains were adapted to growth in Vero cells and were plaque-purified prior to use. Virus pools were prepared in Vero cells and were filtered (0.45 μm Millipore filter) prior to use. All cell lines and virus pools were tested routinely and found free of mycoplasma or other contaminants. Extracts of uninfected Vero cells were prepared for use as controls. Virus titrations by plaque assay were carried out in Vero cells under a methylcellulose overlay.

UV-Irradiation. Five milliliters of filtered HSV or Vero cell extract, placed in a 100 mm open petri dish, was irradiated on a horizontal shaker using two Sylvania G15T8 germicidal lamps set at a distance to give 50 ergs (5 μJ)/mm^2 per sec.

Antisera. The preparation in rabbits and the characterization of antisera against HSV and infectious bovine rhinotracheitis virus has been described (15). Human sera positive and negative for HSV neutralizing antibodies were selected by screening.

Rabbit antiserum to Vero cells was prepared by 5 weekly intravenous injections of 10^7 cells. Preimmunization serum showed no cytotoxic activity at a 1:10 dilution, while hyperimmune serum killed >80% of the Vero cells at a 1:50 dilution in the presence of human complement.

Goat antiserum to mouse xenotropic type C virus, strain ATS 124, was obtained from Dr. Raymond Gilden (Flow Laboratories). Anti-ATS 124 serum does not neutralize rat leukemia virus.

All sera were heat-inactivated at 56° for 30 min and sterilized by filtration prior to use.

Assay for Type C Virus Activation. Sensitive techniques have been developed for studying activation of endogenous type C viruses using cells transformed by sarcoma virus in the absence of helper leukemia virus (NP cells). Activation of type C leukemia virus in NP cells results in the synthesis of sarcoma virus containing the coat proteins of the activated virus (5, 16). The sarcoma virus produced can be assayed

Abbreviations: HSV, herpes simplex virus; NP, nonproducer; MOI, multiplicity of infection; IdUrd, 5-iododeoxyuridine; BrdUrd, 5-bromodeoxyuridine; NRK, normal rat kidney cells.

Table 1. Activation of endogenous type C virus in K-BALB and 43-2 cells by HSV and drugs

Treatment	Virus induction frequency					
	Exp. 1	Exp. 2*	Exp. 3*	Exp. 4	Exp. 5	Exp. 6
Cells	K-BALB	K-BALB	K-BALB	K-BALB	43-2	43-2
Controls						
Medium	$<5.0 \times 10^{-6}$	$<3.5 \times 10^{-6}$	$<1.0 \times 10^{-6}$	$<5.0 \times 10^{-6}$	$<5.0 \times 10^{-6}$	3.3×10^{-6}
Dexa	5.0×10^{-6}		$<1.0 \times 10^{-6}$		$<5.0 \times 10^{-6}$	
IdUrd	3.1×10^{-2}				2.0×10^{-2}	
IdUrd + Dexa	2.8×10^{-2}				3.0×10^{-2}	
Cyclo	3.0×10^{-3}	3.3×10^{-3}	5.8×10^{-3}		5.5×10^{-4}	
Cyclo + Dexa	2.1×10^{-2}		5.5×10^{-2}		6.1×10^{-3}	
Virus†						
HSV-1	3.5×10^{-5} (MOI = 9)		3.1×10^{-5} (MOI = 12)	1.9×10^{-4} (MOI = 36)	2.5×10^{-5} (MOI = 14)	2.0×10^{-4} (MOI = 62)
HSV-1 + Dexa	5.5×10^{-5} (MOI = 9)		4.5×10^{-5} (MOI = 12)		1.5×10^{-5} (MOI = 14)	
HSV-2	3.5×10^{-5} (MOI = 9)	3.2×10^{-5} (MOI = 12)		2.3×10^{-4} (MOI = 32)	4.5×10^{-5} (MOI = 12)	3.7×10^{-4} (MOI = 53)
HSV-2 + Dexa	3.5×10^{-5} (MOI = 9)				3.5×10^{-5} (MOI = 12)	

K-BALB and 43-2 cultures were inoculated with either UV-irradiated (6–10 min) HSV or control fluids (medium alone or UV-irradiated or nonirradiated Vero cell extracts) and incubated for 2 hr. The cells were washed and refed, and drugs were added where indicated. The drugs tested included IdUrd (20 μg ml^{-1}), cycloheximide (Cyclo, 20 μg ml^{-1}), and dexamethasone (Dexa, 0.1 μg ml^{-1}). The cells were incubated for 18–24 hr, treated with mitomycin, and trypsinized. Appropriate cell numbers were inoculated on NRK indicator cells for determination of sarcoma virus induced infectious centers. Each experiment was run in duplicate or triplicate, and the results shown represent the average for each condition tested.

* Carried out by S.A.A.

† MOI determined prior to UV-irradiation.

for focus forming units on permissive indicator cells (17).

Two endogenous type C viruses have been activated in BALB/c cells. BALB:virus 1, activated by IdUrd (18), is classified as N-tropic (19), and BALB:virus 2, activated by IdUrd or inhibitors of protein synthesis (20), is classified as xenotropic (21).

The methods used for assaying type C virus induction were essentially as described (17). Nonproducer K-BALB and 43-2 cells seeded 24 hr prior to use were inoculated with filtered UV-irradiated HSV or control fluid. The multiplicity of infection (MOI) was calculated based on the HSV titers determined in Vero cells prior to UV-irradiation. The infected NP cells were incubated at 37° for 2 hr, washed with medium, and refed; drugs were added where indicated. The cultures were incubated at 37° for appropriate periods. Mitomycin (25 μg ml^{-1}) was added; the cells were incubated for 1 hr and washed three times with medium. The cells were refed, incubated 1–2 hr, trypsinized, and counted by hemocytometer.

Indicator NRK, BALB/c, or NIH Swiss cells were seeded 24 hr prior to use at a concentration of 1.0 to 1.5 $\times$ 10^5 in 60 mm petri dishes in medium containing polybrene (2 μg ml^{-1}) (22). The medium was removed, appropriate volumes of filtered (0.45 μm) supernatant fluids or mitomycin treated NP cells were added, and the cultures were incubated at 37°. The indicator cells were refed on day 3–4 and the number of foci determined after 7–10 days by microscopic examination. The cells in representative cultures were fixed with methanol and stained with Giemsa to verify the results with unfixed cells.

RESULTS

Type C Virus Activation by HSV. Activation of type C virus in K-BALB and 43-2 cells by UV-irradiated HSV and by various drugs was determined by infectious center formation in NRK cells. A total of 15 experiments were carried out in this series, of which six are summarized in Table 1. Table 2 shows the complete data from one experiment.

The three HSV strains tested showed comparable levels of type C virus activation with K-BALB and 43-2 cells. The spontaneous levels of activation with both cells was $\leq 5 \times 10^{-6}$ regardless of whether medium alone or UV-irradiated or nonirradiated Vero cell extracts were used as controls.

Table 2. Activation of endogenous type C virus in K-BALB cells by HSV-2

Treatment	Infectious centers (virus induction frequency)	
	10^5 Cells	10^4 Cells
Controls		
Medium	0, 0, 0, 0 ($<2.5 \times 10^{-6}$)	0, 0, 0, 0 ($<2.5 \times 10^{-5}$)
Vero extract	0, 0, 0, 1 (2.5×10^{-6})	0, 0, 0, 0 ($<2.5 \times 10^{-5}$)
HSV-2*		
MOI = 75	13, 24, 17, 12 (1.7×10^{-4})	0, 1, 1, 3 (1.2×10^{-4})
MOI = 7.5	0, 1, 1, 4 (1.5×10^{-5})	0, 0, 0, 0 ($<2.5 \times 10^{-5}$)

Filtered extracts of uninfected or HSV infected Vero cells were UV-irradiated for 9 min and inoculated on K-BALB cells. An additional control culture received medium alone. The cultures were incubated for 2 hr, washed, refed, and incubated for 19 hr. The cells were treated with mitomycin, and 10^5 and 10^4 cells were inoculated on NRK indicator cells for determination of sarcoma virus induced infectious centers.

* MOI determined before UV-irradiation.

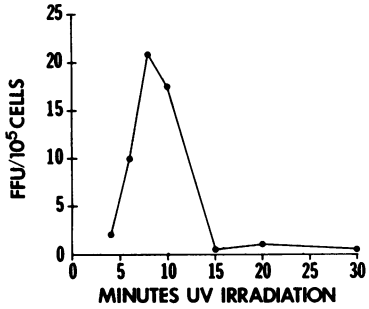

FIG. 1. Effect of time of UV-irradiation of HSV on activation of type C virus. HSV-2 (Savage) UV-irradiated for the times indicated was tested in K-BALB cells for activation of type C virus (see legend to Table 1). Infectious HSV was not detected with samples irradiated for ≥4 min. FFU, focus forming units.

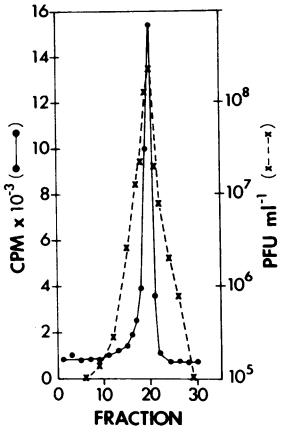

FIG. 2. Purification of HSV-2. Five bottles of Savage HSV [one bottle labeled with 5 μCi ml^{-1} [^{3}H]dT (specific activity 26 Ci mmol^{-1})] were pooled, filtered, treated with DNase (1.5 μg ml^{-1}) and RNase (2.5 μg ml^{-1}), and concentrated with 8% polyethylene glycol 6000. The virus was layered on a 5–35% potassium tartrate gradient (0.01 M Tris, 0.001 M EDTA, pH 7.6) and centrifuged 2 hr at 5° at 25,000 rpm in the SW27 rotor. Fractions were collected from the top. The virus in fraction 20 was tested for activation of type C virus (see Table 3, Exp. 3). PFU, plaque forming units.

When HSV MOI ≈10 was used, the levels of type C virus activation were approximately 10-fold above spontaneous levels, and with MOI >30 the levels progressively increased to approximately 100-fold above background. Infectious center formation by HSV infected NP cells was detected only in NRK cells; foci were not detected in BALB/c or NIH Swiss mouse cells. Sarcoma virus induced infectious centers were not observed when UV-irradiated HSV was inoculated directly in NRK cells.

Treatment with mitomycin could be initiated as early as 2 hr after HSV infection and low levels of type C virus detected. Maximum activation levels were reached by 12 hr after HSV infection, and remained constant for at least an additional 12 hr.

Activation of type C virus by HSV occurred at levels lower than those observed with either IdUrd or cycloheximide. The levels of type C virus activation by HSV and IdUrd were not significantly affected by dexamethasone, while dexamethasone did increase the activation levels with cycloheximide.

Maximum levels of type C virus activation were observed with HSV that had been passaged at low MOI (≤1) in Vero cells. When high MOI (>10) were used, the levels of activation were reduced significantly (data not shown), although the HSV titers were comparable to those obtained with low MOI. Additional studies (data not shown) indicated that HSV passaged at high MOI contained varying amounts of DNA which banded at a higher than normal density in CsCl, suggesting the presence of defective particles (23). Although inconclusive, these findings suggest that defective HSV particles may not efficiently activate type C virus in mouse cells.

Finally, the time of UV-irradiation of HSV for type C virus activation was limited to approximately a 6- to 10-min period (Fig. 1). Irradiation for periods <4 min usually resulted in residual infectious HSV with concomitant cell killing. Infection with HSV irradiated for periods ≥6 min did not adversely affect the viability or the ability of K-BALB or 43-2 cells to replicate, as determined by cell counts made at the time of mitomycin treatment 18–24 hr after infection. During this 18- to 24-hr period, the cell population doubled at least one time.

Effect of Purification and Neutralization of HSV on Activation of Type C Virus. Filtered preparations of HSV or control fluids were treated at 36° for 1 hr with either anti-HSV serum or control serum at a final concentration of 10% (vol/vol). In some experiments the sera were reacted with virus that had been treated with DNase and RNase and purified in a potassium tartrate gradient (Fig. 2). The control sera tested included normal rabbit (preimmune) serum, antiserum against infectious bovine rhinotracheitis virus, anti-Vero cell serum, human serum, and anti-ATS 124 serum. The treated samples were UV-irradiated and tested for their ability to activate type C virus. The results of three of five experiments are summarized in Table 3.

None of the control sera lacking HSV neutralizing antibodies affected the ability of HSV (purified or nonpurified) to activate type C virus in either K-BALB or 43-2 cells. In contrast, neutralization of HSV to low levels (residual MOI <0.1) completely eliminated the virus' ability to activate type C virus. Partial neutralization of HSV resulted in reduced levels of type C virus activation which were comparable to the levels expected for the MOI used (data not shown). Finally, inactivation of HSV by heating at 56° for 1 hr also eliminated the virus' ability to activate type C virus (data not shown).

Persistence of Type C Virus Synthesis in HSV Infected Cells. Replicate cultures of K-BALB cells were inoculated with either medium alone or with UV-irradiated HSV. The cells and medium were tested daily for sarcoma virus (Table 4). Maximum virus production was observed on day 1 with both the cells and medium, although the virus titers in the medium were relatively low. Production of sarcoma virus returned to control cell levels within 3–4 days.

Characterization of Type C Virus Activated by HSV. NRK cultures infected with sarcoma virus from HSV activated K-BALB or 43-2 cells were passaged to enhance the virus titers. The sarcoma virus titers in the filtered medium ranged from $10^{1.5}$ to $10^{2.5}$ focus forming units ml^{-1}. Electron microscopic examination indicated typical type C particles in NRK cultures inoculated with cell-free virus from HSV infected NP cells, while control cultures were negative.

The sarcoma virus isolated from K-BALB or 43-2 cells by HSV types 1 and 2 and propagated in NRK cells was characterized in three ways. First, the virus was filtered and dilutions were inoculated on polybrene treated NRK, BALB/c, and NIH Swiss cells. The BALB/c and NIH Swiss cells showed no foci, even when the cells were passaged two to three times. In contrast, the sarcoma virus titers in NRK cells ranged from $10^{0.5}$ to $10^{2.5}$ focus forming units ml^{-1}. Second,

Table 3. Effect of neutralization of HSV on activation of endogenous type C virus

Treatment	Virus induction frequency		
	Exp. 1	Exp. 2*	Exp. 3†
Cells	K-BALB	K-BALB	43-2
Controls			
Medium	5.0×10^{-6}	4.2×10^{-6}	$<5.0 \times 10^{-6}$
Vero + NS	5.0×10^{-6}		5.0×10^{-6}
Vero + anti-HSV	$<5.0 \times 10^{-6}$		$<5.0 \times 10^{-6}$
Virus‡			
HSV-1 + NS	1.9×10^{-4} (MOI = 35)	1.2×10^{-4} (MOI = 108)	1.7×10^{-4} (MOI = 55)
HSV-1 + anti-HSV	5.0×10^{-6} (MOI = 0.005)	2.7×10^{-6} (MOI = 0.01)	$<5.0 \times 10^{-6}$ (MOI = 0.006)
HSV-2 + NS			7.3×10^{-5} (MOI = 14)
HSV-2 + anti-HSV			2.5×10^{-6} (MOI = 0.008)

Filtered extracts of uninfected or HSV infected Vero cells were treated for 60 min at 36° with either normal serum (NS) or anti-HSV serum (see *text*). The mixtures were UV-irradiated (6–10 min) and inoculated on K-BALB or 43-2 cells. Additional control cultures were incubated for 2 hr, washed, refed, and incubated for 18–24 hr. The cells were treated with mitomycin and trypsinized, and appropriate cell numbers were inoculated on NRK indicator cells for determination of sarcoma virus induced infectious centers. Each experiment was run in duplicate, and the results shown represent the average for each condition tested.

* Carried out by S.A.A.

† Viruses treated with DNase, RNase, and purified in potassium tartrate gradient. See Fig. 2.

‡ MOI determined prior to UV-irradiation.

filtered virus was reacted for 1 hr at 36° with various antisera. Treatment with rabbit or human anti-HSV sera (heat-inactivated) at a final concentration of 10% did not affect the focus forming activity, while anti-ATS 124 serum (heat-inactivated) at a 1:50 dilution completely neutralized the sarcoma virus. Finally, infected NRK cells were extracted with Tween-ether and tested by immunodiffusion with sera specific for mouse and rat type C virus p30 antigens (24). The cell extracts showed a band of identity with mouse but not rat antigen.

Based on the above characteristics, the type C virus activated from K-BALB and 43-2 cells by HSV was classified as a mouse xenotropic virus.

DISCUSSION

The findings reported here indicate that UV-irradiated HSV types 1 and 2 can activate an endogenous mouse xenotropic type C virus. Although the levels of type C virus activated by HSV were relatively low and detection required sensitive techniques, the results proved reproducible in two laboratories.

That activation of type C virus was due to HSV was indicated by the following findings. First, the ability to activate type C virus was eliminated by heat-inactivation or by neutralization of the HSV with specific antiserum. Second, purification of HSV did not affect its ability to activate type C virus. Finally, UV- or non-UV-irradiated extracts of uninfected Vero cells did not induce type C virus when added to NP cells.

Activation of type C virus from K-BALB cells by HSV showed similarities to activation by IdUrd and cycloheximide (18, 20, 25), yet HSV was not similar in all respects to either drug. HSV and cycloheximide were similar in that both activate only xenotropic virus, and with both relatively low levels of sarcoma virus are released into the medium. HSV and IdUrd were similar in that dexamethasone does not enhance the levels of infectious center forming cells.

The findings reported here raise the question of whether the putative oncogenic potential of HSV may involve alterations in the expression of type C virus information. The fact that UV-irradiated HSV can activate an endogenous mouse type C virus is certainly not evidence, *a priori*, of a cofactor role for HSV in oncogenesis. It is of interest, although possibly fortuitous, that the UV inactivation curve for HSV-2 to effect type C virus activation (Fig. 1) and the number of infectious center forming cells observed with low MOI ($\approx$10) of HSV-2 (Table 1) corresponded to the UV inactivation curve and the number of transformed foci observed with comparable MOI in transformation of Swiss/3T3 cells by HSV-2 (26). Recent findings with Marek's disease virus also suggest that infection with this herpesvirus may cause alterations in expression of avian type C virus information (W. Campbell, Life Sciences Inc., personal communication).

Although we cannot equate the finding that HSV can activate type C virus with a cofactor role for HSV in oncogenesis, the findings reported here do suggest possibilities that must be considered in future studies. First, a possible role for type C virus in HSV-associated transformation cannot be excluded simply because type C virus or antigens may not have been detected in transformed cells tested at various times after HSV infection (26). As shown here, synthesis of

Table 4. Time course of type C virus synthesis after activation of K-BALB cells by HSV-2

Treatment	Virus induced cells/10^6 cells and virus released/10^6 cells							
	Day 1		Day 2		Day 3		Day 4	
	Cells	Super	Cells	Super	Cells	Super	Cells	Super
Control	5.0	1.8	5.0	1.2	2.5	0.1	<2.5	<0.1
HSV-2	160.0	23.8	60.0	1.7	4.0	1.3	<2.5	<0.1

Replicate cultures of K-BALB cells were inoculated with either medium alone (control) or with UV-irradiated HSV-2 at a MOI of 52. The cells were incubated for 2 hr, washed, refed, and incubated. The cells in one flask of each were refed daily, and after 24 hr the medium was filtered and inoculated on NRK indicator cells for determination of virus release. The cells were refed, treated with mitomycin, and trypsinized, and 10^5 cells were inoculated on NRK indicator cells for determination of sarcoma virus induced infectious centers. Super = supernatant.

type C virions may occur relatively rapidly after HSV infection and persist for only a short period. Consequently, if testing for type C virus in HSV infected cultures is delayed, one may prematurely conclude from negative findings that type C virus could not have played a role in the initial transformation process. Second, the levels of type C virus production in HSV infected cells may be so low as to make detection difficult, and any virus produced may be of a xenotropic type, which would preclude infection of contiguous cells and resultant virus amplification. Under these conditions, attempts to detect type C virus in the HSV infected cultures by assaying for cell-associated antigens or the presence of RNA-containing particles in the medium may prove difficult, if not impossible.

Finally, the ability of HSV to activate type C virus synthesis may represent only a small part of the total affect HSV plays in altering the expression of type C virus information. It is known, for example, that both "normal" and transformed cells may contain type C virus coded antigens and/or transcripts in the absence of virion formation (27–32). Consequently, HSV infection may have a much larger affect on type C virus expression than indicated by the relatively few cells that show synthesis of complete virions.

Note Added in Proof. Preliminary studies indicate that mouse type C virus can also be activated in BALB/3T3 cells by UV-irradiated HSV. Consequently, the presence in cells of a rescuable sarcoma virus is not required for activation of type C virus by HSV.

We thank Dr. Stephen Oroszlan for carrying out immunodiffusion studies and Mr. Richard DelGuidice for mycoplasma testing. We also thank Drs. Raymond Gilden, Masakazu Hatanaka, and Stephen Oroszlan for many helpful discussions during the course of these studies. This work was supported in part by Contracts NO1-CP-53529 and NCI-E-73-3212 of the Virus Cancer Program of the National Cancer Institute, National Institutes of Health.

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