

Radiometric Detection of Herpes Simplex Viruses

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Two radiometric techniques were investigated as possible means of detecting viruses in clinical specimens. The effect of herpes simplex virus on DNA synthesis by monolayers of human embryonic lung fibroblasts was monitored by the incorporation of [³H] thymidine or [¹²⁵I] iododeoxyuridine ([¹²⁵I] IdU). Radiometric results were compared with those obtained from visual examination for cytopathic effects in the same cell line. Cells infected with herpes simplex virus type-2 (HSV-2, 10^{6.8} virions) showed a marked increase in [³H] thymidine incorporation 2–6 hr after infection. Types 1 and 2 herpes simplex virus yielded similar results, with increased incorporation of tracer being observed 72 hr after infection with 10 virions. The [³H] thymidine technique was used successfully to assay mouse brains infected with HSV-1. Increased [¹²⁵I] IdU incorporation was observed 6 hr after infection with 10⁵–10^{6.8} HSV-1 virions, 24 hr after 10⁵ virions, 48 hr after 10⁵ virions, and 72 hr after 10–100 virions. The increased [¹²⁵I] IdU incorporation was completely inhibited by preneutralization with immune serum. These radiometric techniques for detection of viral effect on cellular metabolism are simple, objective, and quantitative.

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Viral identification in clinical and epidemiologic investigations is usually done directly by isolation or indirectly by serologic methods (1–3). Both methods are time-consuming, with the former requiring highly skilled personnel. Recent effort has focused on the development of radioimmunoassays for the quantification of viral antigens and antibodies (5–7). Few studies have reported the use of radioactive tracer principles for the assay of biologically active viruses (8–10).

We have previously developed a simple radiometric technique for rapid detection of herpes simplex virus type 1 (HSV-1) in WI-38 cell culture (11). Our studies are based on the hypothesis that (a) early metabolic effects of viruses on cell culture can be used as an indication of viral presence; (b) specificity can be achieved by neutralization of viral effects with specific antiserum; and (c) radiometric techniques can be used to measure these virus-induced metabolic effects. In the present work we further extended our study of the rapid detection of HSV-1, first by using the [³H] thymidine-incor-

poration technique to detect a closely related virus, HSV-2; second, by attempting to increase sensitivity with iododeoxyuridine (IdU) pretreatment; and, third, by investigating the possible extension to clinical material using a HSV-1 infected mouse-brain model. Also described is a new technique using [¹²⁵I] iododeoxyuridine ([¹²⁵I] IdU) for prolonged, non-destructive monitoring of HSV activity in cell culture.

MATERIALS AND METHODS

Cells. WI-38 and MRC-5 cells in 23rd to 26th passage were seeded as described previously (11) at a concentration of 2×10^5 cells/ml. Cells were grown 4 days in (a) Basal Medium Eagle (Modified) with Earl's salts, (b) 10% fetal calf serum, (c) 25 mM hepes buffer, and (d) 100 units potassium penicillin G, 100 μ g streptomycin, and 100 μ g kana-

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mycin per milliliter (BME₉₀ FC₁₀). Cells were then changed to Minimal Essential Medium Eagle (Modified) with Earl's salts, 3% fetal calf serum and antibiotics (MEM₉₇ FC₃), and were used the following day. The [³H] thymidine incorporation technique used 1-dram culture vials containing 0.5 ml cell suspension. Culture vials routinely yielded 1–2 × 10⁵ cells/monolayer as determined by direct counting in a hemocytometer. The [¹²⁵I] IdU incorporation technique used 16- × 125-mm glass culture tubes containing 1 ml cell suspension. Culture tubes yielded approximately 3.7 × 10⁵ cells/monolayer.

Virus. HSV-1 stock was prepared and titrated as previously described (11) with a titer of 5.6 × 10⁷ TCID₅₀/ml. (One TCID₅₀ (Tissue Culture Infective Dose) represents the dose that gives rise to cytopathic changes in 50% of the inoculated cultures.) HSV-2 stock (MS Strain, ATCC 540) was prepared in a similar manner with a similar titer. Virus stock was diluted in MEM₉₇FC₃ for dose-response determination. Then, 0.1 ml of each dilution was added per vial. Samples were prepared in triplicate and assayed 6, 24, 48, and 72 hr after infection.

The radiometric techniques for viral detection were compared with visual detection of histologic evidence of cell damage (cytopathic effects, CPE), in corresponding test-tube monolayers maintained in triplicate, as previously reported (11).

IdU pretreatment. WI-38 cells were allowed to grow 1 day and were then treated with 0.5 ml of BME₉₀FC₁₀ containing IdU (10 μg/ml) for 4 days. IdU-treated and untreated control cells were washed twice with 1 ml MEM₉₇FC₃. Cells were infected with HSV-1 at varying multiplicities of infection, and HSV-1 activity was monitored by the [³H] thymidine incorporation technique.

Animal studies. Encephalitis was produced in weanling mice (male, Swiss) by intracerebral inoculation of 0.03 ml HSV-1 (3.2 × 10⁴ TCID₅₀/ml). Control mice received 0.03 ml of phosphate-

buffered saline (PBS). The majority of virus-infected mice began to exhibit symptoms 3 days after inoculation. Infected and uninfected brains were harvested 4 days after inoculation, weighed, and washed three times with PBS. They were then placed in vials containing 1 ml MEM₉₇FC₃ and stored at -70°C. The following day the brains were homogenized with tissue grinders after the addition of a second milliliter of MEM₉₇FC₃. The homogenates were centrifuged at 950 g for 45 min at 4°C and the supernatant was used for cell inoculation.

[³H] thymidine-incorporation technique. Measurement of [³H] thymidine incorporation was performed as described previously (11). Briefly, confluent monolayers were infected with 0.1 ml of virus stock or clarified tissue homogenates. Control cells received 0.1 ml of MEM₉₇FC₃. Inoculated cells were incubated at 37°C for 60 min, followed by addition of 0.5 ml/vial of MEM₉₇FC₃ containing 1 μCi of [methyl-³H] thymidine (45 Ci/mM). At the designated time, H-3-labeled medium was aspirated and the cell monolayer was washed twice with 2 ml MEM₉₇FC₃. This procedure removed all the extracellular radioactivity without disrupting the monolayer (11). Caps of the washed vials were discarded and vials placed in 20-ml glass liquid scintillation vials followed by the addition of 15 ml of Bray's solution. Samples were counted with a liquid-scintillation spectrometer.

[¹²⁵I] IdU-incorporation technique. After decanting of the overlying medium, confluent monolayers were infected with 0.1 ml of virus stock, at a virus-to-cell ratio of approximately 15. One-tenth milliliter of MEM₉₇FC₃ was added to control cells. After 60 min, 1 ml of MEM₉₇FC₃ per vial was added, followed by 1 μCi of [5-¹²⁵I] iododeoxyuridine (2,000 Ci/mM) in 0.1 ml MEM₉₇FC₃. Cells were incubated at 37°C. Preliminary experiments indicated that material of lower specific activity was not as sensitive. At the designated time, I-125-labeled medium was

TABLE 1. EFFECT OF HSV-2 ON [³H] THYMIDINE INCORPORATION BY WI-38 CELLS

	Time (hours after infection)					
	2		4		6	
	Mean	Range	Mean	Range	Mean	Range
Control*	16,652	11,649–20,500	25,617	15,552–41,556	33,385	23,983–52,553
HSV-2	37,192	26,891–61,067	112,834	94,775–175,356	177,841	182,891–227,667
% Control	218	178–302	432	226–609	565	436–763
p value	<0.05		<0.025		<0.01	

* Results are expressed as mean and range (cpm). Each of four experiments was run in triplicate and the results averaged. The number of WI-38 cells used was 1 × 10⁶, and the quantity of HSV-2 was 5.6 × 10⁶ TCID₅₀.

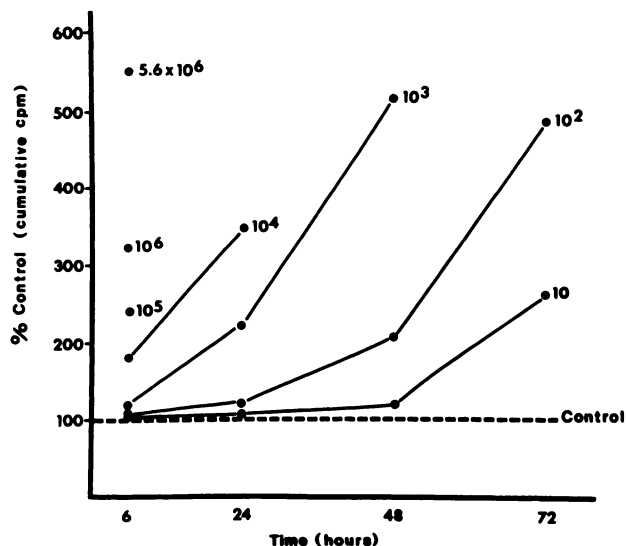


FIG. 1. Effect of varying numbers of HSV-2 virions on $[^3\text{H}]$ thymidine incorporation by WI-38 cells. Results are expressed as percentage of control (control = 100%) for triplicate samples from a typical experiment. Number of WI-38 cells used was 100,000. Number of virions used ranged from 10 to 5.6 million TCID₅₀, as indicated.

decanted, the remaining monolayer washed twice with 4 ml of MEM₉₇FC₃, 1 ml of unlabeled medium added, and tubes counted with a scintillation spectrometer. Tubes were relabeled by addition of 1 $\mu\text{Ci}/0.1$ ml $[^{125}\text{I}]$ IdU, and incubated until the time for subsequent measurements. Samples were prepared in quintuplicate; radioactive measurements were obtained at 6, 24, 48, and 72 hr after infection.

Dose-response determination was performed as previously described, except that samples were prepared in quintuplicate. Viral neutralization tests were performed as previously described (11) using herpes simplex human immune serum.

Statistics. The calculation of statistical significance was based on pair differences (12).

RESULTS

Effect of HSV-2 on $[^3\text{H}]$ thymidine incorporation. We have previously shown that HSV-1 stimulates DNA synthesis by WI-38 cells (11). To confirm that the same system is applicable to HSV-2, its effect on $[^3\text{H}]$ thymidine incorporation was studied. As shown in Table 1, HSV-2 (5.6×10^6 TCID₅₀) stimulated $[^3\text{H}]$ thymidine incorporation by WI-38 cells. In virus-infected cells, there was a twofold increase in incorporation beginning 2 hr after infection, proceeding to fourfold by 4 hr and sixfold by 6 hr.

Figure 1 shows the effect of various multiplicities of infection of HSV-2 on $[^3\text{H}]$ thymidine incorporation. The level of sensitivity is a function of time: by 72 hr after infection an initial inoculum of even 10 virions is detectable. This sensitivity is similar to that for HSV-1. Attempts to distinguish between Type 1 and Type 2 have failed because human immune sera inhibited the stimulation of $[^3\text{H}]$ thymidine incorporation by both HSV-1 and HSV-2 due to cross reactivity of the antisera.

Effect of IdU pretreatment on $[^3\text{H}]$ thymidine incorporation. Green and Baron (14) report that 5-iododeoxyuridine (IdU) potentiates the in vitro replication of several unrelated RNA and DNA viruses. Accordingly the effect of pretreatment with IdU on $[^3\text{H}]$ thymidine incorporation by uninfected and HSV-1-infected WI-38 cells was investigated to determine whether detection time and/or sensitivity could be improved. IdU-pretreated cells incorporated approximately one-half the amount of $[^3\text{H}]$ thymidine as did untreated cells, and exhibited a 10% reduction in cell number. Pretreatment did not decrease the detection time or increase the sensitivity, for treated and untreated cells yielded similar degrees of stimulation of $[^3\text{H}]$ thymidine incorporation at each time interval after infection.

HSV-1-infected mouse-brain model. Experimen-

TABLE 2. DETECTION OF HSV-1 IN INFECTED MOUSE BRAINS BY MEASUREMENT OF $[^3\text{H}]$ THYMIDINE INCORPORATION INTO WI-38 CELLS

Clinical symptoms*	No. of mice	% control (20 hr after infection)†	First appearance of CPE (hr)	TCID ₅₀ /0.1 ml brain homogenate‡
Severe	5	606 (564-645)	20-36	>10 ⁴
Minor	5	238 (213-260)	48-60	4.9 × 10 ³ (3.2-5.6 × 10 ³)
None	4			
	(2)	124 (121-127)	72	5.6 × 10 ²
	(2)	108 (103-112)	neg	neg

* Severe: tremors, contralateral hemiplegia; Minor: lethargic, ruffled fur.

† Results are mean cumulative cpm, expressed as percentage of control plus range. Quadruplicate samples were run, and the results averaged, for each infected mouse. Control values represent means of five uninfected mice. Each brain was run in quadruplicate.

‡ Results are expressed as mean and range of designated number of mice, each run in triplicate.

Effect of Varying Numbers of HSV-1 on ¹²⁵I-IdU Incorporation by WI-38 Cells

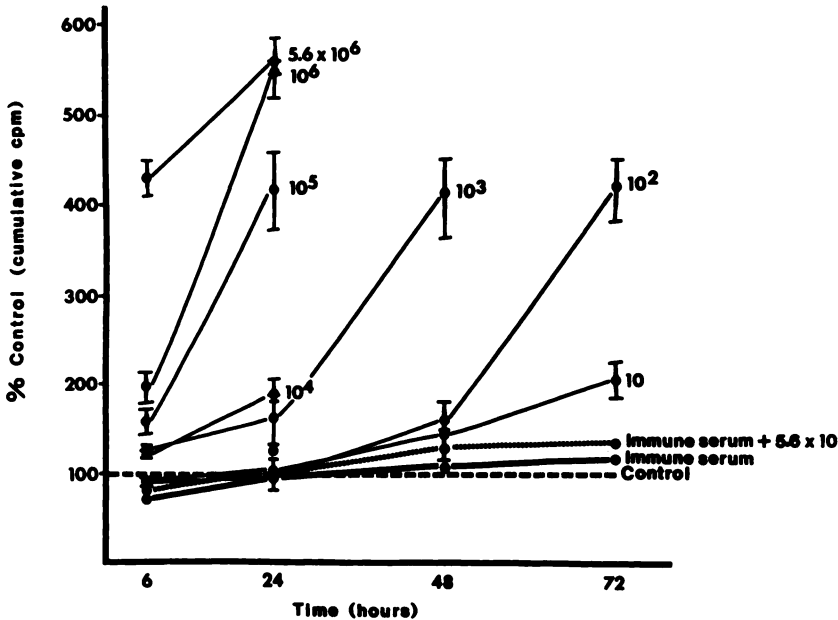


FIG. 2. Effect of varying numbers of HSV-1 virions, and neutralization by human immune serum, on [¹²⁵I] IdU incorporation by WI-38 cells. Results are expressed as percentage of control (mean and range). Each of three experiments was done in quintuplicate. The number of WI-38 cells used was approximately 3 × 10⁵. Points designating viral neutralization are expressed as percentage of control for quintuplicate samples from a typical experiment.

tally induced HSV-1 encephalitis in weanling mice was employed to ascertain whether the [³H] thymidine-incorporation technique has any possible efficacy in the detection of HSV in clinical specimens. Table 2 illustrates the detection of HSV-1 in mouse brains by the measurement of [³H] thymidine incorporation into WI-38 cells. With brains from mice exhibiting minor to severe symptoms, significant in-

corporation was observed. Increased [³H] thymidine incorporation was detected approximately 1 day before visual signs of CPE with material from mice with minor symptoms. Uninfected mouse-brain homogenates had no significant effect on [³H] thymidine incorporation. Such material gave incorporation values almost identical to those of controls—i.e., for cells inoculated with 0.1 ml MEM₉₇FC₈.

Effect of HSV-1 on [¹²⁵I] IdU incorporation. The incorporation of [¹²⁵I] IdU, a thymidine analogue, into infected and uninfected cells was also investigated. It can be measured nondestructively without addition of scintillation fluid, allowing repetitive measurements on the same sample. Figure 2 shows the dose-response effect of HSV-1 on [¹²⁵I] IdU incorporation by WI-38 cells. Increased incorporation was observed 6 hr after infection with 10⁵–10^{6.8} virions (p < 0.005), 24 hr after 10⁴ virions (p < 0.005), 48 hr after 1,000 virions (p < 0.001), and 72 hr 10–100 virions (p < 0.01, p < 0.005, respectively). These radiotracer measurements were not always more rapid than the appearance of early signs of characteristic CPE (Table 3).

Since the availability of WI-38 cells is uncertain at present (14), another well-characterized human embryonic lung-fibroblast cell line, MRC-5, was evaluated. Similar results were obtained with HSV-1 infected MRC-5 cells. With the concentration of [¹²⁵I] IdU used, no gross toxicity to either cell line was observed.

TABLE 3. EFFECT OF VARYING NUMBERS OF HSV-1 VIRIONS ON APPEARANCE OF CPE IN WI-38 CELLS

	Time (hours after infection)			
	6	24	48	72
Control	—	—	—	—
10 ^{6.8}	+ ⁰	4 ⁺	—	—
10 ⁶	+ ⁰	4 ⁺	—	—
10 ⁵	—	3 ⁺	4 ⁺	—
10 ⁴	—	1 ⁺	3 ⁺	—
10 ³	—	+	2 ⁺	4 ⁺
10 ²	—	—	1 ⁺	3 ⁺
10	—	—	+ ¹	+ ⁵

* Results are expressed as mean values of nine experiments, each run in triplicate.

- 4⁺ = 75–100% monolayer affected.
 - 3⁺ = 50–75% monolayer affected.
 - 2⁺ = 25–50% monolayer affected.
 - 1⁺ = 1–25% monolayer affected.
 - +⁰ = 10 foci (+¹ = 1 focus, +⁵ = 5 foci).
 - +⁰ = generalized early CPE.
- Similar results were obtained with HSV-2.

Specificity was achieved by neutralization of viral effects with specific antiserum. Neutralization of varying numbers of HSV-1 virions by human immune serum resulted in complete elimination of the increased [¹²⁵I] IdU incorporation observed when HSV-1 alone was present (Fig. 2). Immune serum alone did not cause any increase in incorporation. Viral neutralization was confirmed by absence of CPE up to 2 wk after inoculation.

DISCUSSION

The data presented here further substantiate our previous conclusion (11) that radiometric measurements of the effects of HSV on DNA synthesis by cultured cells can be used as a quantitative and objective assay for biologically active virus. Specificity can be afforded by neutralization of the virus with immune serum. Sensitivity is time-dependent, an initial inoculum of 10 virions being detectable by 72 hr after infection. The measurements are at least as fast, if not more rapid, than the appearance of characteristic cytopathic effects. The [³H] thymidine technique was shown to be efficacious for the assay of HSV-1 in infected mouse brains. Smith and Melnick (15) have reported that the concentration of HSV in vesicular fluid ranges from 3×10^9 virions/ml to 7×10^{10} /ml. Thus it would probably be possible to detect HSV from vesicle fluid with our radiometric technique in a few hours. Uninfected mouse-brain homogenates, immune serum, and bacteria (11) exhibited no effect on DNA synthesis by infected or control cells. Thus, these techniques may be applicable to certain clinical specimens.

The [³H] thymidine-incorporation method is a destructive technique, since addition of scintillation fluid terminates the incubation. Thus it requires multiple samples for measurement at different time intervals. The available quantity of a clinical specimen is often insufficient to allow multiple samplings. The [¹²⁵I] IdU-incorporation technique has distinct advantages over the [³H] thymidine technique because it is nondestructive, thus allowing repeated measurements over extended periods and requiring a minimal quantity of sample. This would be particularly important in application of this technique to clinical samples where the amount of the virus is unknown. IdU is known to act as a thymidine analog and therefore is initially incorporated into DNA both in mammalian cells (16) and in viruses (17). However, it is not a perfect analog, and in subsequent replication steps it inhibits DNA synthesis in cells (18) and viruses (19). IdU also inhibits a role in phenomena that are less well understood. It is reported to enhance the replication of several unrelated viruses (13), to convert nonpermissive cells to a permissive

state (20), and to activate RNA (21) and DNA (22) tumor viruses. In this study we showed that pretreatment with IdU did not increase the sensitivity of HSV detection by [³H] thymidine incorporation but that [¹²⁵I] IdU could be employed as a simple, quantitative, and objective assay of a DNA virus such as herpes simplex. The efficacy of these radiometric techniques in diagnostic virology, and in monitoring noncytopathic viruses and viruses which do not multiplying cell cultures, awaits further evaluation.

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