jnm / IN VITRO NUCLEAR MEDICINE

Simple Radiometric Techniques for Rapid Detection of Herpes Simplex Virus Type 1 in WI-38 Cell Culture

Nancy D'Antonio, Min-Fu Tsan, Patricia Charache, Steven Larson, and Henry N. Wagner, Jr.

The Johns Hopkins Medical Institutions, Baltimore, Maryland

Two radiometric techniques were developed for detecting the presence of herpes simplex virus type 1 in stationary monolayers of the diploid cell line WI-38. The time of detection was compared to that obtained from visual examination for cytopathic effects in the same cell line. Oxidation of ^{14}C-1-glucose in infected and uninfected cells was determined by $^{14}CO_2$ production, measured by an ionization chamber, and DNA synthesis was determined by ^{3}H-thymidine incorporation, measured by scintillation counting. Compared to uninfected cells, infected cells showed a 23–26% reduction in ^{14}C-1-glucose oxidation and a 355–498% increase in DNA synthesis 4–6 hr after infection. These changes in cellular metabolism were observed 14 hr before visible signs of cytopathic effects. The increase in DNA synthesis was completely inhibited by viral neutralization with herpes simplex antiserum. Increased DNA synthesis was observed 5 hr after infection with 10,000–3,200,000 $TCID_{50}$ units of virus. These radiometric methods for the detection of viral effect on cellular metabolism are simple, fast, and objective.

J Nucl Med 17: 503–507, 1976

While detecting bacteria is routine in clinical medicine, detecting viruses remains difficult and is not as widespread as is warranted by the frequent occurrence of viral infections. Current immunofluorescent (*1,2*) and immunoenzymatic (*3,4*) techniques are rapid but require carefully controlled conditions and experienced personnel. A simple, rapid, and objective method of detecting viral activity would have an obvious advantage in diagnostic virology.

Numerous biochemical changes and functional disturbances precede any visible histologic changes and cell death in cultures infected with a cytocidal virus such as herpes simplex type 1 (HSV-1) (*5*). Microscopic evidence of cellular infection (cytopathic effect) is the diagnostic criterion most commonly employed in the clinical laboratory to detect the presence of viruses (*6,7*). We hypothesized that early biochemical effects of viral growth in cell culture can be used to detect viral presence; this approach has been successfully employed to detect bacterial growth (*8,9*). Infection of human embryonic lung fibroblasts (WI-38 cells) by HSV-1 was chosen as the test model. In this study we report the radiometric measurement of the effects of HSV-1 on glucose oxidation and nucleic acid synthesis by WI-38 cells during the early hours of infection before any visible signs of abnormal cell function are apparent.

MATERIALS AND METHODS

Cells. We planted WI-38 cells in the 20th to 24th passages (HEM Research, Inc., Rockville, Md.) at a concentration of 100,000 cells/ml in a total volume of 2 ml. The cells were grown 2–4 days as stationary monolayers in Eagle's basal medium, consisting of Earle's base plus 10% fetal calf serum, 25 m*M* hepes buffer, and 100 units of potassium penicillin G, 100 μg of streptomycin, and 100 μg of kanamycin per mil-

Received Nov. 4, 1975; revision accepted Dec. 29, 1975.

For reprints contact: Min-Fu Tsan, Johns Hopkins Medical Institutions, 615 N. Wolfe St., Baltimore, Md. 21205.

liliter. The glucose oxidation method used sterile 10-ml serum vials with rubber liners (Johnston Laboratories, Cockeysville, Md.) and aluminum airtight seals (Wheaton Scientific, Millville, N.J.) for cell cultivation, the vials being incubated in a horizontal position at 37°C. The nucleic acid synthesis method used autoclaved 20-ml glass liquid-scintillation vials with nontoxic screw caps (Wheaton Scientific) incubated in a vertical position. Both cell systems routinely yielded 300,000–400,000 cells/monolayer, determined by direct cell counting as follows: the monolayer was trypsinized with 1 ml of 0.25% trypsin–0.02% EDTA (Gibco, Grand Island, N.Y.), neutralized with 0.9 ml of medium, stained with 0.1 ml of a 0.4% trypan blue aqueous solution, and counted by means of a counting chamber.

Virus stock. A patient isolate of HSV-1 was obtained from the Johns Hopkins Hospital Virology Laboratory. Stock virus was prepared in WI-38 monolayers maintained on Eagle's minimal essential medium, Earle's base plus 3% fetal calf serum with buffer, and antibiotics as listed. The viral material was frozen and thawed twice and then stored at −70°C. The stock was sterility-tested and assayed for glucose concentration. The titer was determined by tube titration in WI-38 cells, and the 50% endpoint was calculated by the method of Reed and Muench (*10*), yielding 3,200,000 $TCID_{50}$* per 0.1 ml. Tube titration was confirmed by plaque assay (*11*), which yielded 7 million plaque-forming units† per 0.1 ml.

Glucose assay. Virus stock was mixed with an equal amount of 10% trichloroacetic acid and then centrifuged at 200 g for 20 min. The supernatant was used to measure the glucose concentration by a hexokinase–glucose–6-phosphate dehydrogenase method utilizing an automatic clinical analyzer (Dupont Instruments, Newton, Conn.). The glucose concentration of the virus stock was found to be 36 ± 1 mg%.

Glucose oxidation system. Serum vials containing preconfluent monolayers with overlay medium removed were infected with 0.1 ml of virus stock, at a virus-to-cell ratio of approximately 10. Uninfected cells received 0.1 ml of an equalized glucose solution prepared in Earle's basal salts. Virus was exposed to the cells for 60 min at 37°C with mild agitation at 15-min intervals. Following the adsorption period, 1.9 ml of low-glucose maintenance medium (i.e., the maintenance medium without the usual 0.1% glucose) plus 4 μCi (60 mCi/m*M*) of ^{14}C-1-D-glucose (Amersham/Searle Corp., Arlington Heights, Ill.) was added per vial. All vials were prepared in quintuplet. Background controls consisted of an equal volume of ^{14}C-labeled medium without cells or virus.

Measurement of glucose oxidation. The $^{14}CO_2$ produced by cellular metabolism was measured with Bactec R-301 (Johnston Laboratories, Inc.). The $^{14}CO_2$ produced from ^{14}C substrate in the culture vial was flushed into the ionization chamber through two needles inserted in the septum and measured as ionic current. This measured radioactivity was expressed in index units (I.U.), where 100 I.U. $\simeq$ 0.025 μCi. The atmosphere in the vial was replaced with 10% CO_2 culture gas. The $^{14}CO_2$ production from infected and uninfected cells was monitored at 2, 4, 6, 24, 48, and 72 hr after the ^{14}C-labeled medium was added.

Nucleic acid synthesis system. Stationary monolayers were infected as in the glucose oxidation system. One-tenth milliliter of phosphate-buffered saline (PBS) was added to the uninfected control cells. After the adsorption period, 1.9 ml of maintenance medium plus 1 μCi of ^{3}H-methylthymidine (52 Ci/m*M*) or ^{3}H-5,6-uridine (40 Ci/m*M*) (Amersham/Searle Corp.) was added to each infected and control vial. Background controls consisted of equal amounts of ^{3}H-labeled medium without cells or virus. All samples were prepared in duplicate and taken for measurement at 1, 2, 4, and 6 hr after infection.

Viral neutralization tests were performed as follows: herpes simplex human immune serum (Flow Laboratories, Rockville, Md.) was heat-inactivated at 56°C for 30 min, mixed with an equal amount of the virus stock, and incubated at 37°C for 60 min, and then 0.2 ml of the mixture was inoculated. Samples were assayed at 1, 2, 4, and 6 hr after infection.

Virus stock was diluted in maintenance medium to contain $\log_{10}$ quantities for dose–response determination. Then, 0.1 ml of the appropriate dilution was added per vial, and uninfected control cells received 0.1 ml of maintenance medium. In this group of experiments, the cells were cultured in one-dram vials (Wheaton Scientific), yielding 40,000 cells per vial as compared to ~ 400,000 cells per vial in previous experiments. Samples were prepared in triplicate and assayed 5 hr after infection.

Measurement of nucleic acid synthesis. The amount of ^{3}H-thymidine or ^{3}H-uridine incorporated by the WI-38 cells was measured by liquid-scintillation counting. At the designated time intervals, the ^{3}H-labeled medium was aspirated and the remaining cell monolayer (or empty vial in case of background controls without cells) was washed twice with 10 ml of cold PBS. In preliminary experiments, direct cell

* One $TCID_{50}$ (Tissue Culture Infective Dose) represents the dose that gives rise to cytopathic changes in 50% of the inoculated cultures.

† One plaque-forming unit represents a circumscribed area of cellular degeneration initially produced by one virion.

TABLE 1. EFFECT OF HSV-1 ON ^{14}C-1-GLUCOSE OXIDATION BY WI-38 CELLS

	Time after infection (hr)					
	2	4	6	24	48	72
Control*	58 ± 8	226 ± 36	438 ± 74	1,222 ± 203	1,874 ± 253	2,310 ± 253
HSV-1*	44 ± 8	173 ± 33	326 ± 33	720 ± 125	1,078 ± 168	1,322 ± 187
% Control	83.4	76.6	74.4	59.0	57.5	57.2
p value	<0.01	<0.01	<0.005	<0.01	<0.005	<0.005

* The results are expressed as mean ± standard error (index units) of the cumulative $^{14}CO_2$ production. Each of four experiments was done in quintuplet and the results were averaged. The number of WI-38 cells used was 300,000–400,000, and the quantity of HSV-1 was 3,200,000 $TCID_{50}$ units.

TABLE 2. EFFECT OF HSV-1 ON DNA SYNTHESIS BY WI-38 CELLS

	Time after infection (hr)			
	1	2	4	6
Control*	2,958 ± 678 (4)	3,700 ± 447 (4)	5,818 ± 685 (5)	7,245 ± 720 (3)
HSV-1*	2,789 ± 479 (4)	8,341 ± 1,930 (4)	26,482 ± 3,392 (5)	43,298 ± 7,882 (3)
% Control	94.3	225.4	455.2	597.6
p value	>0.5	>0.05	<0.005	<0.05

* The results are expressed as mean ± standard error (cpm). Each experiment was done in duplicate and the results averaged; the number in parenthesis indicates the number of experiments. The number of WI-38 cells used was 300,000–400,000, and the quantity of HSV-1 was 3,200,000 $TCID_{50}$ units.

TABLE 3. EFFECT OF HSV-1 ON RNA SYNTHESIS BY WI-38 CELLS

	Time after infection (hr)			
	1	2	4	6
Control*	5,242 ± 2,475	10,086 ± 4,324	17,007 ± 8,498	28,328 ± 11,607
HSV-1*	5,305 ± 2,503	10,158 ± 4,610	20,274 ± 9,392	30,351 ± 13,603
% Control	101.2	100.7	119.2	107.1
p value	>0.5	>0.5	>0.1	>0.4

* The results are expressed on the same basis as in Table 2. Three experiments were performed.

counting indicated that this washing procedure did not detach cells; it did remove all of the extracellular radioactivity, as measured with ^{14}C-inulin. Samples were prepared for liquid-scintillation counting by the addition of 0.4 ml of 0.5 *M* Protosol (New England Nuclear, Boston, Mass.) for 10 min at 37°C, followed by 15 ml of Bray's solution. Samples were counted with a Tri-Carb scintillation spectrometer Model 3003 (Packard Instrument Co., Downers Grove, Ill.).

Examination for cytopathic effects. Radiometric viral detection in both systems was compared with visual detection by typical cytopathic effects (CPE) in WI-38 test-tube monolayers maintained in triplicate. After removal of the overlay medium, virus was inoculated and tubes placed on a roller for 60 min at 37°C. One milliliter of maintenance medium was then added, and microscopic examination was performed at comparable time intervals until a positive CPE was identified.

Sterility testing. Sterility checks of random samples from each experiment were performed on chocolate agar and in Schaedler's broth, and radiometrically with ^{14}C-U-glucose aerobic and anaerobic culture vials (Johnston Laboratories, Inc.).

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

RESULTS

Effect of HSV-1 on glucose oxidation. The effect of HSV-1 on ^{14}C-1-glucose oxidation by WI-38 cells is shown in Table 1. As early as 2 hr after infection

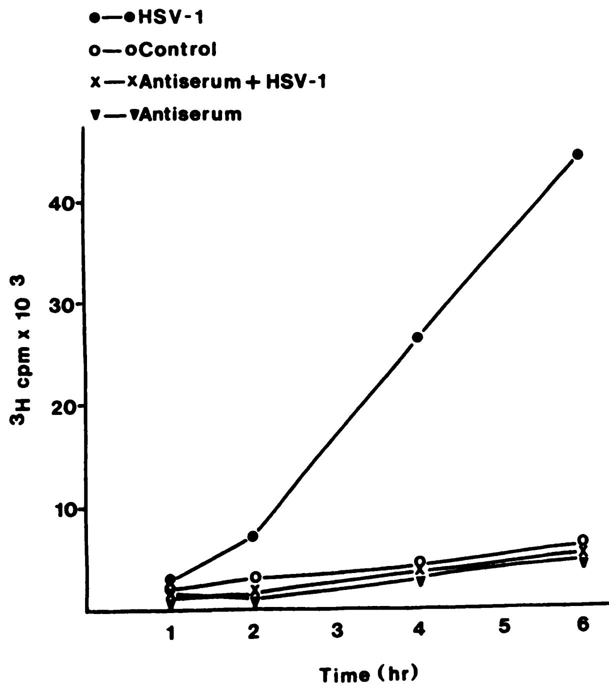

FIG. 1. Effect of HSV-1 neutralization by specific human immune serum on DNA synthesis by WI-38 cells. Each point represents mean of duplicate samples from typical experiment.

there was a significant depression of glucose oxidation by cells infected with HSV-1. This effect was observed 16 hr before visual signs of CPE. The degree of the inhibition of glucose oxidation by HSV-1 infection continued to increase up to 72 hr (17% at 2 hr and 43% at 72 hr).

Effect of HSV-1 on nucleic acid synthesis. Table 2 shows the effect of HSV-1 on DNA synthesis by WI-38 cells. In virus-infected cells there was a marked stimulation of DNA synthesis: at 4 hr after infection, there was a fourfold stimulation. This was at least 14 hr before any signs of CPE were visible. In contrast, HSV-1 had no effect on RNA synthesis by WI-38 cells (Table 3). Thus, the tremendous stimulation of DNA synthesis by this DNA virus was not accompanied by a comparable stimulation in RNA synthesis.

Adding 10,000 bacteria of three different bacterial species (*Staphylococcus epidermidis, Pseudomonas aeruginosa*, and *Acinetobacter calcoaceticus* var. *anitratus*) to each sample had no effect on the DNA synthesis of infected or control cells.

Effect of viral neutralization with specific antiserum on DNA synthesis. Neutralization of HSV-1 with specific antiserum resulted in complete elimination of the stimulation of DNA synthesis observed when HSV-1 is present alone (Fig. 1). Antiserum alone had no effect on DNA synthesis by WI-38 cells. Neutralization of virus with antiserum was confirmed by the absence of CPE over a 3-day period.

Effect of various numbers of HSV-1 on DNA synthesis. In order to determine the relative sensitivity of the nucleic acid synthesis system, various numbers of virions ranging from 10 to 3,200,000 $TCID_{50}$ units were tested. Since preliminary experiments indicated that an increase in the multiplicity of infection by a decrease in the number of WI-38 cells increases the degree of stimulation of DNA synthesis, monolayers composed of only 40,000 WI-38 cells were used for this part of the study. As shown in Table 4, a significant stimulation of DNA synthesis by WI-38 cells could be detected with 10,000 virions at 5 hr after infection.

DISCUSSION

This study indicates that radiometric measurements of the effect of HSV-1 on the metabolism of tissue culture cells can be used to detect viruses. Moreover, neutralization of virus with specific antiserum can be used for the purpose of speciation. This effect on cellular metabolism could be detected as early as 5 hr after infection of 40,000 WI-38 cells with 10,000 $TCID_{50}$ units of virus. This is at least 13 hr before any evidence of visible cytopathic effects.

TABLE 4. EFFECT OF VARIOUS NUMBERS OF HSV-1 ON DNA SYNTHESIS BY WI-38 CELLS 5 HR AFTER INFECTION

	Infectivity ($TCID_{50}$/sample)*						
	3.2×10^6	10^6	10^5	10^4	10^3	10^2	10^1
% Control	844 ± 79 (3)	741 ± 12 (3)	437 ± 65 (4)	198 ± 14 (4)	117 ± 17 (4)	126 ± 7 (3)	125 ± 17 (4)
p value	<0.025	<0.001	<0.025	<0.01	>0.2	>0.2	>0.2

* The results are expressed as mean percent of control ± standard error (cpm). Each experiment was done in triplicate and the results were averaged; number in parenthesis indicates the number of experiments. The number of WI-38 cells used was 40,000.

The sequence of replication and biosynthesis of HSV and its effect on host macromolecular synthesis have been well documented (*13,14*). Synthesis of host DNA ceases completely within 3–5 hr after infection, indicating that the large increase of DNA synthesis observed in HSV-1-infected cells is due to the replication of the viral genome. Only about 20% of the viral DNA is found in infectious particles (*13*). Thus, the measurement of nucleic acid synthesis would seem more sensitive than methods that depend on the presence of the infectious virion.

The early inhibition of ^{14}C-1-glucose oxidation by HSV-1 in WI-38 cells has not been previously demonstrated. Graves (*15*), utilizing a poliovirus–HeLa-cell system, was unable to detect any effect on glucose oxidation. Our method of measuring glucose oxidation by monitoring $^{14}CO_2$ release with an ionization chamber has the advantages of being nondestructive and of allowing repeated sampling from the same vial over extended periods. However, the presence of a relatively high concentration of glucose in biologic fluid, such as blood, poses a practical problem, since this nonlabeled glucose would compete with ^{14}C-glucose for oxidation.

In the nucleic acid synthesis system, the cells were grown in liquid-scintillation counting vials. This allowed us to perform the entire procedure, from infection with virus to the final quantification of DNA synthesis, in the same vial. These radiometric techniques for the early detection of HSV-1 are simple and objective. Further study is necessary to assess these techniques in the actual detection and identification of the virus in clinical specimens.

ACKNOWLEDGMENTS

This work was supported by contracts from U.S. Army Medical Research and Development Command, DAMD 17-74-C-4057 and 17-75-C-5041, and USPHS Research Grant GM-10548.

REFERENCES

1. Hanna L, Keshishyan H, Jawetz E, et al: Diagnosis of *Herpesvirus hominis* infections in a general hospital laboratory. *J Clin Microbiol* 1: 318–323, 1975

2. Nahmias AJ, Shore SL, DelBuono I: Diagnosis by immunofluorescence of human viral infections with emphasis on herpes simplex viruses. In *Viral Immunodiagnosis.* New York, Academic, 1974, pp 157–172

3. Hahon N, Simpson J, Eckert HL: Assessment of virus infectivity by the immunofluorescent and immunoperoxidase techniques. *J Clin Microbiol* 1: 324–329, 1975

4. Morisset R, Kurstak C, Kurstak E: Diagnosis of herpes simplex virus infections with immunoperoxidase. In *Viral Immunodiagnosis.* New York, Academic, 1974, pp 31–40

5. Smith JD, deHarven E: Herpes simplex virus and human cytomegalovirus replication in WI-38 cells. I. Sequence of viral replication. *J Virol* 12: 919–930, 1973

6. Cooney MK: Relative efficiency of cell cultures for detection of viruses. *Health Lab Sci* 10: 294–302, 1973

7. Joseph JM: Laboratory diagnosis of viral diseases. *Md State Med J* 20: 63–69, 1971

8. DeBlanc HJ, DeLand FH, Wagner HN: Automated radiometric detection of bacteria in 2,967 blood cultures. *Appl Microbiol* 22: 846–849, 1971

9. Larson SM, Charache P, Chen M, et al: Automated detection of *Haemophilus influenzae. Appl Microbiol* 25: 1011–1012, 1973

10. Reed LJ, Muench HA: A simple method for estimating fifty percent endpoints. *Am J Hyg* 27: 493–497, 1938

11. Wentworth BB, French L: Plaque assay of *Herpesvirus hominis* on human embryonic fibroblasts. *Proc Soc Exp Biol Med* 131: 588–592, 1969

12. Croxton FE: Reliability and significance of arithmetic means. In *Elementary Statistics with Applications in Medicine and the Biological Sciences.* New York, Dover, 1959, pp 240–242

13. Nahmias AJ, Roizman B: Infection with herpes simplex viruses 1 and 2 (first of three parts). *N Engl J Med* 289: 667–674, 1973

14. Nahmias AJ, Roizman B: Infection with herpes simplex viruses 1 and 2 (third of three parts). *N Engl J Med* 289: 781–789, 1973

15. Graves IL: Incorporation of radioactivity from ^{14}C-sugars into macromolecules in poliovirus-infected or guanidine-treated HeLa cells. *J Gen Virol* 4: 101–109, 1969

CAP QUALITY ASSURANCE PROGRAM INVITES PARTICIPATION

At the Board of Trustees Meeting in January, a resolution submitted by N. E. Herrera, M.D., of the College of American Pathologists (CAP) was presented to the SNM Quality Control Committee by its chairman, William J. MacIntyre, Ph.D. The Advisory Committee of CAP's Quality Control Phantom Project announced its interest in the development of an intersocietal committee on quality control, requested that the various societies contacted urge their members to participate in the program, and recommended that the Phantom Survey Project continue under the administration of CAP. The Quality Control Committee of SNM is pleased to inform Society members of the availability of this quality assurance program and will explore further the possibilities of participating in an intersocietal committee of this nature. Please address all inquiries to: **N. E. Herrera, M.D., Director, Dept. of Nuclear Medicine, Danbury Hospital, Danbury, Conn. 06810.**