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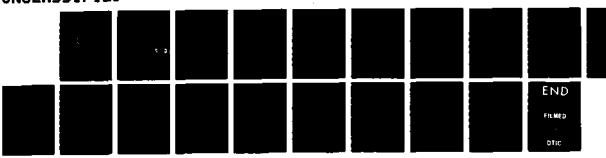

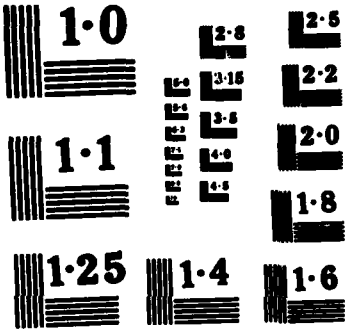

RADIOMETRIC METHODS FOR RAPID DIAGNOSIS OF VIRAL INFECTION

Annual Report

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Two radiometric techniques were investigated for monitoring the effect of herpes simplex virus on the DNA synthesis of monolayers of human embryonic lung fibroblasts. DNA synthesis of infected and uninfected cells was monitored by ^{3}H-thymidine incorporation measured by liquid scintillation counting or by ^{125}I-iododeoxyuridine (^{125}I-IdU) incorporation measured non-destructively by gamma scintillation counting. Radiometric results were compared to those obtained from visual examination for cytopathic effects in the same cell line. Herpes simplex virus type-2 (HSV-2, ~~$10^{6.8}$~~ $10^{6.8}$ $TCID_{50}$) infected cells showed

10 to the 6.8th power

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20. a marked increase in ^{3}H-thymidine incorporation 2-6 hr after infection. HSV-2 and herpes simplex type 1 (HSV-1) exhibited similar levels of sensitivity with increased incorporation being observed 72 hr after infection with 10 virions. The ^{3}H-thymidine technique was utilized to assay HSV-1 infected mouse brains. Increased ^{125}I-IdU incorporation was observed 6 hr after infection with ~~10^{5}-$10^{6.8}$~~ HSV-1 virions, 24 hr with ~~10^{4}~~ virions, 48 hr with ~~10^{3}~~ virions, and 72 hr with 10-10^{2} virions. The increased ^{125}I-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.

10 to the 5th power to 10 to the 6.8th power

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INTRODUCTION

Direct laboratory isolation of virus is important for the understanding of clinical and epidemiological characteristics of viral diseases (1,2). Although there are many techniques for the detection of viruses (1,3), clinical diagnosis of viral infection continues to be difficult and impractical. With the promise of antiviral agents for the treatment of viral infection (4), the importance of early diagnosis and identification of the virus become more apparent. Radioactive tracers are among the most sensitive substances detectable by modern technology. Recent effort has focused on the development of radioimmunoassays for the quantification of viral antigens and antibodies (5-7). Few studies have been done utilizing radioisotopes 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 1) early metabolic effects of virus on the cell culture can be used as an indication for the presence of virus; 2) the specificity can be achieved by neutralization of viral effects with specific antiserum; and 3) radiometric technique can be used to measure these metabolic effects of virus. In this report we further extended our study on the rapid detection of HSV and described a non-destructive method utilizing ^{125}I-iododeoxyuridine (^{125}I-IdU) for prolonged, uninterrupted monitor of HSV activity in cell culture.

Materials and Methods

Cells. WI-38 and MRC-5 cells in 23rd to 26th passage (HEM Research, Inc., Rockville, Md.) were seeded as described previously (11) at a concentration of 2 x 10^5 cells/ml. Cells were grown 4 days in Basal Medium Eagle (Modified) with Earl's salts

(Flow Lab., Rockville, Md.), 10% fetal calf serum, 25 mM hepes buffer, and 100 units potassium penicillin G, 100 ug streptomycin, and 100 ug kanamycin per milliliter. Cells were then changed to Minimal Essential Medium Eagle (Modified) with Earl's salts (Flow Lab.), 3% fetal calf serum and antibiotics ($MEM_{97}FC_3$), and were used the following day. The ^{3}H-thymidine incorporation technique utilized 1 dram culture vials (Wheaton Scientific, Millville, NJ) containing 0.5 ml cell suspension. Culture vials routinely yielded 1-2 x 10^5 cells/monolayer as determined by direct counting in a hemocytometer. The ^{125}I-IdU incorporation technique used 16 x 125 mm glass culture tubes (Corning Glass Works, Corning, NY) containing 1 ml cell suspension. Culture tubes yielded approximately 3.7 x 10^5 cells/monolayer.

Virus stock. HSV-1 stock was prepared and titrated as previously described (11) with a titer of 5.6 x 10^7 $TCID_{50}$*/ml. HSV-2 stock (MS strain, ATCC 540) was prepared in a similar manner with a titer of 5.6 x 10^7 $TCID_{50}$/ml.

^{3}H-thymidine incorporation technique. Measurement of ^{3}H-thymidine incorporation was performed as described previously (11). Briefly, confluent monolayers with overlay medium aspirated were infected with 0.1 ml of virus stock or suspected virus infected material. Uninfected control cells received 0.1 ml of $MEM_{97}FC_3$. Inoculated cells were incubated at 37°C for 60 min, followed by addition of 0.5 ml/vial of $MEM_{97}FC_3$ containing 1 uCi of ^{3}H-methylthymidine (45Ci/mM, Amersham/Searle Corp., Arlington Heights, Ill). The amount of ^{3}H-thymidine incorporated by the cells was measured by liquid scintillation counting. At the designated time, ^{3}H-labeled medium was aspirated and the remaining cell monolayer was washed twice with 2 ml $MEM_{97}FC_3$. This procedure removed all the

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

extracellular radioactivity and did not disrupt the monolayer (11). Caps of the washed vials were discarded and vials placed in 20-ml glass liquid scintillation vials (Wheaton Scientific) followed by the addition of 15 ml of Bray's solution. Samples were counted with a Tri-Carb scintillation spectrometer Model 3003 (Packard Instrument Co., Downers Grove, Ill).

Virus stock was diluted in $MEM_{97}FC_3$ to contain log_{10} quantities 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.

HSV-1 infected mouse brain model. Encephalitis was produced in weanling mice (male, Swiss, Buckberg Co., Tomkinson Cove, NY) by intracerebral inoculation of HSV-1 (9.6×10^2 $TCID_{50}$/0.03 ml). Control mice received 0.03 ml of phosphate buffered saline (PBS). Majority of mice began to exhibit symptoms 3 days after inoculation. Infected and uninfected brains were harvested 4 days after inoculation and were washed in 3 successive 60 mm petri dishes containing PBS. They were then placed in dram vials containing 1 ml $MEM_{97}FC_3$ and stored at -70°C. The following day brains were homogenized. The homogenates were centrifuged at 950 g for 45 min at 4°C. Supernatant was used for the detection of HSV-1 by ^{3}H-thymidine incorporation in WI-38 cells and for quantification by tube titration. The $TCID_{50}$ titers were calculated by the Reed and Muench method (12).

^{125}I-IdU incorporation technique. Confluent monolayers with overlay medium decanted were infected with 0.1 ml of virus stock, at a virus-to-cell ratio of approximately 15. One-tenth milliliter of $MEM_{97}FC_3$ was added to uninfected control cells. After the 60 min adsorption period, 1 ml of $MEM_{97}FC_3$/vial was added, followed by 1 uCi of

5-^{125}I-iododeoxyuridine (2000Ci/mM, New England Nuclear, Boston, Ma) in 0.1 ml per tube. Cells were incubated at 37°C. Preliminary experiments indicated lower specific activity material did not give suitable sensitivity levels.

The amount of ^{125}I-IdU incorporated by the cells was measured by gamma scintillation counting. At the designated time, ^{125}I-labeled medium was decanted, the remaining monolayer washed twice with 4 ml of $MEM_{97}FC_3$, 1 ml unlabeled medium added, and tubes counted with a Auto-gamma scintillation spectrometer Model 5986 (Packard Instrument Co.). Tubes were relabeled by addition of 1 uCi/0.1 ml ^{125}I-IdU, and reincubated until time for subsequent measurements. Samples were prepared in quintuplet and radioactive measurements obtained at 6, 24, 48, and 72 hr after infection.

Dose reponse determination was performed as previously stated, except samples were prepared in quintuplet. Viral neutralization tests were performed as previously described (11) using herpes simplex human immune serum (Flow Lab.).

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

Statistics. The calculation of statistical significance was based on pair differences (13).

RESULTS

Effect of HSV-2 on ^{3}H-thymidine incorporation. We have previously shown that HSV-1 stimulates DNA synthesis by WI-38 cells (11). In order to see whether HSV-2 also stimulates DNA synthesis, its effect on ^{3}H-thymidine incorporation by WI-38 cells was studied. As shown in table 1, HSV-2 (5.6×10^{6} $TCID_{50}$) stimulated ^{3}H-thymidine incorporation by WI-38 cells. In virus-infected cells, there was a 2-fold increase in incorporation beginning 2 hr after infection, proceeding to 4-fold by 4 hr and 6-fold by 6 hr.

Effect of varying numbers of HSV on ^{3}H-thymidine incorporation. Relative sensitivity was determined by addition of decreasing numbers of HSV-2 virions ranging from $10^{6.8}$ to 10 $TCID_{50}$ doses. As shown in Fig. 1, the level of sensitivity is a function of time, and by 72 hr after infection even an initial inoculum of 10 virions is detectable. HSV-1 exhibited a similar level of sensitivity. Attempts to distinguish between type 1 and type 2 have failed due to inability to obtain non-cross-reacting antisera.

Effect of IdU pretreatment on ^{3}H-thymidine incorporation. It has been reported that 5-iododeoxyuridine (IdU) potentiates the *in vitro* replication of several unrelated RNA and DNA viruses (14). Thus, the effect of pretreatment with IdU on ^{3}H-thymidine incorporation by HSV-1 infected and uninfected WI-38 cells was investigated to determine if detection time and/or sensitivity could be improved. WI-38 cells were pretreated with 0.5 ml of IdU (10 ug/ml) for 4 days. After removal of IdU, cells were infected with HSV-1 and ^{3}H-thymidine incorporation was determined as before. No significant effect was observed (data not shown).

HSV-1 infected mouse brain model. Experimentally induced HSV-1 encephalitis
ı weanling mice was employed to ascertain if the ^{3}H-thymidine incorporation technique
as any possible efficacy in the detection of HSV in clinical specimens. Radiometric
ıeasurement of HSV-1 infected and uninfected mouse brains by ^{3}H-thymidine incorporation
y WI-38 cells is shown in Table 2. Significant incorporation was observed with mice
xhibiting minor to severe symptoms. Increased ^{3}H-thymidine incorporation was
etected approximately 1 day before visual signs of CPE with material from mice with
ıinor symptoms. Uninfected mouse brain homogenates had no effect on ^{3}H-thymidine
ıcorporation.

Effect of varying numbers of HSV-1 on ^{125}I-IdU incorporation. The ^{3}H-thymidine
ıcorporation method is a disruptive technique, since addition of scintillation fluid terminates
he experiment. Thus, it requires multiple samples for measurement at different time
ntervals. The available quantity of a clinical specimen is often insufficient to allow
ıultiple samplings. Therefore, the incorporation of ^{125}I-IdU, a thymidine analogue,
ıto infected and uninfected cells was investigated. ^{125}I is a gamma emitter. It can
e counted non-destructively without addition of scintillation fluid, allowing repetative
ıeasurements on the same sample. Fig. 2 shows the dose response effect of HSV-1 on
^{25}I-IdU incorporation by WI-38 cells. Increased incorporation was observed 6 hr after
ıfection with 10^{5}-$10^{6.8}$ virions (p 0.005), 24 hr with 10^{4} virions (p 0.005), 48 hr with
0^{3} virions (p 0.001), and 72 hr with 10-10^{2} virions (p 0.01, p 0.005). These
adiometric measurements were not always more rapid than the appearance of early signs
f characteristic CPE (Table 3).

Effect of viral neutralization with immune serum on ^{125}I-IdU incorporation.

utralization of varying numbers of HSV-1 with human immune serum resulted in complete mination of the increased ^{125}I-IdU incorporation observed when HSV-1 alone is present able 4). Immune serum alone did not cause any increase in incorporation. Viral utralization was confirmed by absence of CPE over a 2 week period.

Since the availability of WI-38 cells may be in jeopardy (15), another well aracterized human embryonic lung fibroblast cell line, MRC-5, was evaluated. Similar sults were obtained with HSV-1 infected MRC-5 cells.

DISCUSSION

The data presented in this report further substantiates our previous conclusion (11) radiometric measurements of the effects of HSV on DNA synthesis by cultured cells be used as a quantitative and objective assay for the active virus. Specificity can be rded by neutralization of the virus with immune serum. Sensitivity is time-dependent ı an initial inoculum of 10 virions being detectable by 72 hr after infection. These ısurements are at least as fast, if not more rapid than the appearance of characteristic ɔpathic effect. The ^{3}H-thymidine technique was shown to be efficious for the assay ISV-1 in infected mouse brains. Smith and Melnick (16) have reported that the centration of HSV in vesicular fluid ranges from 3×10^{9}/ml to 7×10^{10}/ml. Thus, ɜ possible to detect HSV from vesicle fluid with our radiometric technique in a few rs.

The ^{125}I-IdU incorporation technique has distinct advantage over the ^{3}H-thymidine hnique because it is non-destructive, thus allowing repeated measurements over ended periods and requiring a minimal quantity of samples. IdU is known to incor- ate into DNA of mammalian cells (17) and into viruses (18). It has been shown to ibit diverse effects from inhibition of cancer cells (19) and viruses (20) to enhancement ·eplication of unrelated viruses (14), conversion of non-permissive cells to a permissible te (21) and activation of RNA (22) and DNA (13) tumor viruses. The radionuclide I-IdU has been employed in assays for tumor cells (24) and cell-mediated cytotoxicity (25), may exhibit cell toxicity under certain conditions (26). In this study we showed that I-IdU might also be employed as a simple, quantitative and objective assay of a DNA

virus such as herpes simplex.

Radiometric detection of HSV in cell culture as shown in this study depends on the metabolic effects of virus, although the metabolic effects of viruses on cell cultures are closely related to their cytopathic effect, a dissociation of these two effects may occur. Thus, there are viruses, such as West Nile virus, which multiply in the cell culture, but do not produce any cytopathic effect. Our radiometric technique would have an obvious advantage under this situation. There are also some viruses, such as hepatitis B virus, thought to be very difficult or unable to multiple in cell cultures. Are they really unable to grow in cell cultures? Or actually they do grow, but we are unable to detect their growth, because they do not produce any morphological changes. Our radiometric technique may be useful to answer this question.

TABLE 1. EFFECT OF HSV-2 ON ^{3}H-THYMIDINE INCORPORATION BY WI-38 CELLS

	Time (hours after infection)		
	2	4	6
Control*	16,652 ± 3320	25,617 ± 12,007	33,385 ± 16,314
HSV-2	37,192 ± 19,620	112,834 ± 58,190	177,841 ± 65,465
% Control	218 ± 91	432 ± 138	565 ± 128
p Value	< 0.05	< 0.025	< 0.01

* The results are expressed as mean ± standard deviation (cpm). Each of 4 experiments was run in triplicate and the results averaged. The number of WI-38 cells used was 1×10^5, and quantity of HSV-2 was 5.6×10^6 $TCID_{50}$.

Fig. 1

Effect of Varying Numbers of HSV-2 on ^{3}H-Thymidine Incorporation by WI-38 Cells

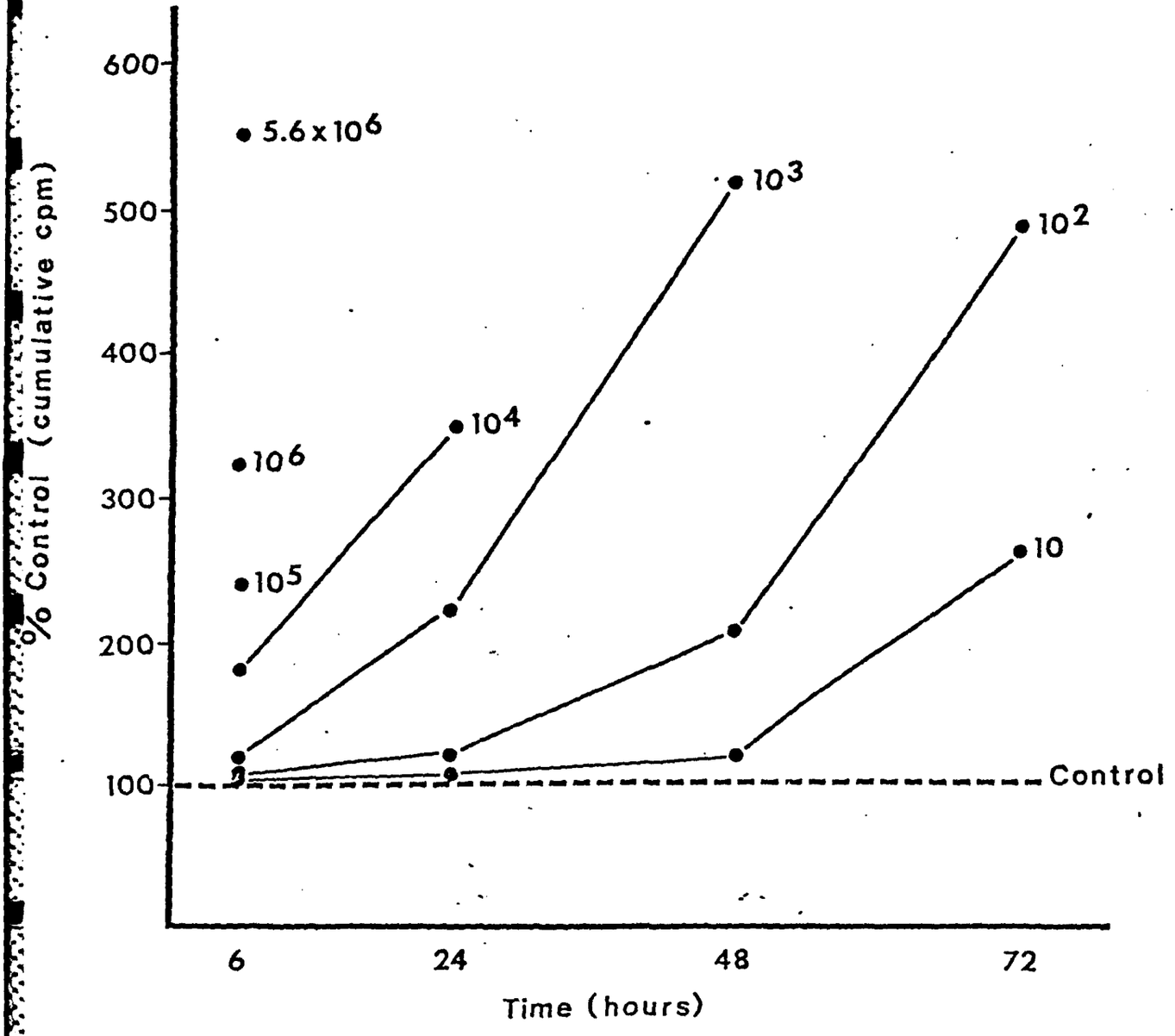

TABLE 2. RADIOMETRIC MEASUREMENT OF HSV-1 INFECTED AND UNINFECTED MOUSE BRAINS BY ^{3}H-THYMIDINE INCORPORATION BY WI-38 CELLS

Mouse No.	%Control (20 hr PI)**	First Appearance of CPE (hr)	$TCID_{50}$/0.1ml Brain Homogenate	$TCID_{50}$/g	Symptoms
1*	645	36-48	$>10^4$	$>10^4$	severe
1	602	20	$>10^4$	$>10^4$	" "
2	582	20	$>10^4$	$>10^4$	" "
3	638	20	$>10^4$	$>10^4$	" "
4	564	36	$>10^4$	$>10^4$	" "
2*	213	60-72	5.6×10^2	1.1×10^4	minor
5	260	48	5.6×10^2	1.1×10^4	" "
6	242	48	4.7×10^2	9.4×10^3	" "
7	223	48	3.2×10^2	6.4×10^3	" "
8	254	48	5.6×10^2	1.1×10^4	" "
3*	112	-	-	-	none
4*	127	72	5.6×10^1	1.1×10^3	" "
9	103	-	-	-	" "
10	121	72	5.6×10^1	1.1×10^3	" "

* Mice from Exp. #1; remaining represent mice from Exp. #2.
** The results are expressed as mean % control (control = 100%) of cumulative cpm. Quadruplet samples were done for each mouse and the results averaged. The control values represent the mean of 5 (Exp.#1) or 10 (Exp.#2) uninfected mouse brains each run in quadruplet.

Fig. 2

Effect of Varying Numbers of HSV-1 on ^{125}IdU Incorporation by WI-38 Cells

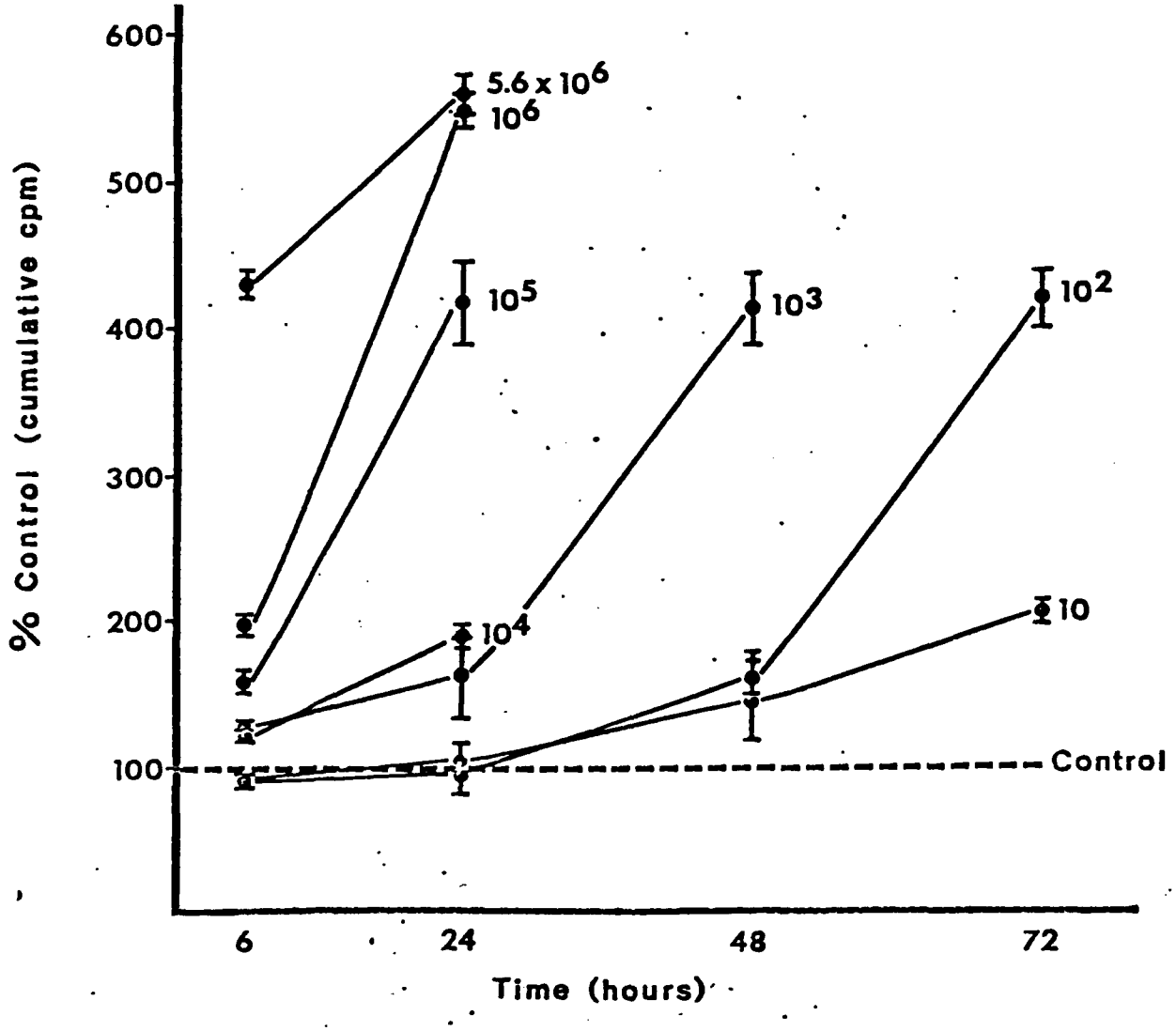

Table 3. EFFECT OF VARYING NUMBERS OF HSV-1 ON APPEARANCE OF CPE BY WI-38 CELLS

	Time (Hours after infection)			
	6	24	48	72
Control*	-	-	-	-
$10^{6.8}$	$+^{o}$	4^{+}		
10^{6}	$+^{o}$	4^{+}		
10^{5}	-	3^{+}	4^{+}	
10^{4}	-	1^{+}	3^{+}	
10^{3}	-	+	2^{+}	4^{+}
10^{2}	-	-	1^{+}	3^{+}
10^{1}	-	-	$+^{1}$	$+^{5}$

* The results are expressed as mean values of 9 experiments, each run in triplicate.

4^{+} = 75-100% monolayer affected
3^{+} = 50-75
2^{+} = 25-50
1^{+} = 1-25
+ = 10 foci ($+^{1}$ = 1 foci, $+^{5}$= 5 foci)
$+^{o}$ = generalized early CPE

TABLE 4. EFFECT OF PRENEUTRALIZATION ON ^{125}I-IDU INCORPORATION BY HSV-1 INFECTED CELLS

	Time (hours after infection)							
	6		24		48		72	
	Virus	Virus + Serum	Virus	Virus + Serum	Virus	Virus + Serum	Virus	Virus + Serum
Control*	100	90	100	140	100	139	100	130
$10^{6.8}$	453	69	538	88		102		101
10^{6}	193	71	538	91		104		103
10^{5}	133	86	439	73		92		102
10^{4}	115	96	207	86	685	103		99
10^{3}	114	68	137	58	467	102		100
10^{2}	120	69	130	70	133	97	410	103
10^{1}	120	91	104	85	130	103	200	98

* The results are expressed as mean values (cpm) based on % contol (control = 100%). The experiment was done in quintuplet and the results averaged.

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