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Chromosomal and Autoradiographic Studies of Cells Infected with Herpes Simplex Virus

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The induction of chromosomal aberrations by herpes simplex virus (HSV) and the interaction between viral deoxyribonucleic acid (DNA) and chromosomes have been studied (i) by infection of the BHK-21 line of hamster kidney cells at multiplicities ranging from 0.5 to 100 followed by 1-hr pulses of ^{3}H-thymidine preceding preparation at varying intervals of metaphases and autoradiography, and (ii) by use of ^{3}H-thymidine-labeled HSV for infection, chromosomal, and autoradiographic analyses at intervals thereafter. The results revealed that (i) chromosomal lesions develop prior to, and thus are independent of, viral DNA synthesis; (ii) HSV is capable of replicating in cells during the late G-2 period or in metaphase; (iii) most of the viral DNA remains unassociated with chromosomes and is not detectable at sites of chromosomal lesions; (iv) the capacity of the virus to cause chromosomal aberrations is four times less sensitive to inactivation by ultraviolet (UV) irradiation than its infectious property; and (v) after large doses of UV, invasion of the nuclei by the irradiated virus is reduced. These observations indicate that the chromosomal lesions induced by HSV result most likely from action of an early enzyme under control of the viral genome. This explanation is proposed also for the effects of adenovirus type 12 on chromosomes.

Rapp and Hsu (10) concluded that viral replication in diploid Chinese hamster cells is necessary for induction of chromosomal aberrations by herpes simplex virus (HSV), since noninfective virus failed to cause such changes. Viral deoxyribonucleic acid (DNA) synthesis could not be demonstrated, however, at or near the areas of chromosomal lesions. In similar studies with adenovirus type 12, chromosomal aberrations were noted in the absence of viral DNA synthesis, suggesting that they were caused most likely by an enzyme under control of the viral genome (6, 7). In view of these apparently discrepant observations, it seemed worthwhile to reexamine HSV-induced chromosomal changes, using the BHK-21 line of Syrian hamster kidney cells. In addition, infection of cell cultures with ^{3}H-thymidine-labeled and partially purified HSV offered a further opportunity to assess interactions between DNA and chromosomes. The results of these studies are summarized in this report.

MATERIALS AND METHODS

Cells and media. The BHK-21 line of Syrian hamster kidney cells was obtained from Microbiological Associates, Inc., Bethesda, Md. The cells were maintained as monolayer cultures in Blake bottles on Basal Medium Eagle (BME) that contained double the amount of vitamins and amino acids. The cultures were supplemented with 10% fetal calf serum (FCS), penicillin (100 units/ml), and streptomycin (100 μg/ml). Cultures were divided every 2 to 3 days, by the use of 0.25% trypsin in Hanks' balanced salt solution to dislodge the cells. Cultures that contained approximately 10^6 cells in the logarithmic growth phase were used for the experiments.

Virus. The H strain of HSV (15) was obtained through courtesy of T. Tokumaru, of the Children's Hospital of Philadelphia. The virus had been passed in primary rabbit kidney (RK) cultures, and a twice plaque-purified stock was prepared and kept at −70 C. Before experimental use, the stock virus was passed once more in RK cultures.

^{3}H-thymidine-labeled virus. Monolayer cultures of about 10^8 primary RK cells were infected with HSV at an input multiplicity of at least 10 plaque-forming units (PFU) per cell. After an adsorption period of 1 hr at 37 C, the cell sheets were washed twice with Hanks' solution and then fed BME that contained 5% dialyzed FCS, and ^{3}H-thymidine at a final concentration of 10 μc/ml. Following further incubation at 37 C for 20 hr, the virus was processed by a minor modification of the method of Levitt and Becker (8). The medium was centrifuged at 35,000 × *g* for 1 hr, and the pellet was resuspended in Ribosome Structure Buffer (16) that contained 2% FCS (RSB-2). The

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suspension was sonically treated for 10 sec (Disintegrator-Forty, Ultrasonic Industries, Plainview, N.Y.) and clarified at 1,000 × *g* for 20 min. The cells of the infected cultures were scraped into 8 ml of RSB-2 and sonically treated for 90 sec; they were then incubated for 30 min at 37 C with 0.4 ml of deoxyribonuclease (0.4 mg), 0.4 ml of ribonuclease (2 mg), and 1 ml of 1 M $MgCl_2$. The sample was clarified at 1,000 × *g* for 20 min. The medium- and cell-derived virus suspensions were pooled and layered on a preformed sucrose gradient (15 to 35%, w/v) in RSB-2. The preparation was subjected to 32,000 × *g* for 40 min in the SW 25.1 rotor of a Beckman L2 preparative centrifuge (Beckman Instruments, Inc., Fullerton, Calif.). Fractions (1-ml) were collected and the five samples with the highest virus concentrations were pooled, dialyzed for 1 hr against phosphate-buffered saline (PBS), and rebanded in a second sucrose gradient. From 3 to 5 fractions of the virus band were pooled and dialyzed against PBS for 20 hr. The titers of the final virus suspensions were of the order of 10^8 PFU/ml. Only 1 to 2% of the radioactivity of these preparations passed through 0.05-μm membrane filters (Millipore Corp., Bedford, Mass.); therefore it was not virion-associated.

Radioactive isotope and scintillation counts. Virus or cells were labeled with ^{3}H-thymidine with specific radioactivity of 6 c/mM (Schwartz Bio Research Inc., Orangeburg, N.Y.). Samples of labeled materials (0.01-ml) were diluted in 10 ml of Bray's buffer (2) and counted in a Tri-Carb liquid scintillation spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.).

Virus titrations. Plaque assays were performed with the fluid overlay technique of Black and Melnick (1). Two RK monolayer cultures in plastic flasks (Falcon Plastics, Inc., Los Angeles, Calif.) were used per virus dilution. After an adsorption period of 1 hr, the cell sheets were washed twice with Hanks' solution and overlayed with BME that had been supplemented with 10% human serum of pools known to contain high titers of neutralizing antibodies of HSV. After incubation for 5 days, the cell sheets were stained with crystal violet (0.05% in 20% ethylalcohol), and the plaques were enumerated. Titers are expressed as PFU/ml. The technique that was used for ultraviolet (UV) irradiation of virus has been fully described (6).

Chromosome preparations and autoradiography. These methods have been recorded in detail (5, 6). Kodak Track Emulsion NTB-3 was used for autoradiography. The exposure time was 2 weeks when labeled virus was used, and 2 days in experiments involving DNA synthesis in infected cells.

RESULTS

Experiments with unlabeled HSV. Chromosomal abnormalities in the form of enhanced secondary constrictions or single chromatid breaks were normally seen in 1 to 3% of BHK-21 cells. In addition to those already mentioned, HSV-induced aberrations consisted of multiple chromatid breaks, complete fragmentation, and stickiness of chromosomes (4, 10, 12). Figure 1 presents the percentages of altered metaphases in relation to the dose of HSV and the time after its inoculation. At an input multiplicity of 50 PFU/cell, all metaphases observed at 6 to 8 hr were abnormal, whereas for 10- and 100-fold lower doses of virus, from 28 to 60% and 12 to 18% of the metaphases, respectively, revealed chromosomal lesions at these times. With production of viral progeny, the percentages of affected metaphases increased further by 24 hr. The numbers of altered chromosomes per metaphase also increased in relation to the dose of virus and the time of incubation.

It is known that the G-2 period of BHK-21 cells is at least 2 hr (3). Detection of label in metaphases after exposure of cultures to a 1-hr pulse of ^{3}H-thymidine in a concentration of 1 μc/ml at any time after infection with HSV should reflect, therefore, viral DNA synthesis; possibly, it could also reflect repair of damaged host cell DNA (11). Accordingly, cultures were infected with 50 or 5 PFU/cell and exposed to ^{3}H-thymidine for 1 hr prior to preparations of metaphases. With the larger dose of virus, 70% of the metaphases were abnormal at 4 hr, but only 2% of these contained a few silver grains. Figure 2 provides examples of metaphases with extensive chromosomal lesions in the absence of label, whereas adjacent nuclei show innumerable silver grains. Labeled metaphases became prominent 6 hr after inoculation of HSV and in the final stages of infection; i.e., at 8 hr after infection with 50 PFU/cell and at 24 hr after infection with 5 PFU/cell, all metaphases were severely damaged and heavily labeled (Fig. 3). The silver grains, as noted by Rapp and Hsu (10), were generally not associated with chromosomes, suggesting that they represented labeled viral DNA.

Experiments with ^{3}H-thymidine-labeled HSV.

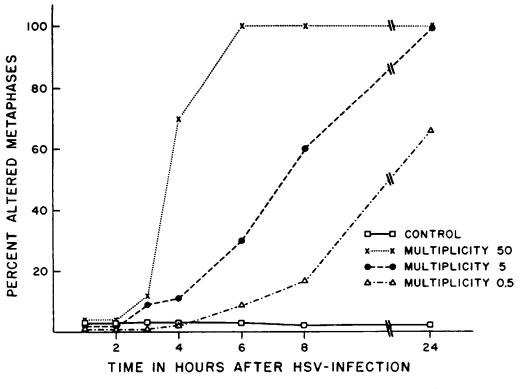

FIG. 1. *Percentages of abnormal metaphases in relation to dose of HSV and to time of incubation.*

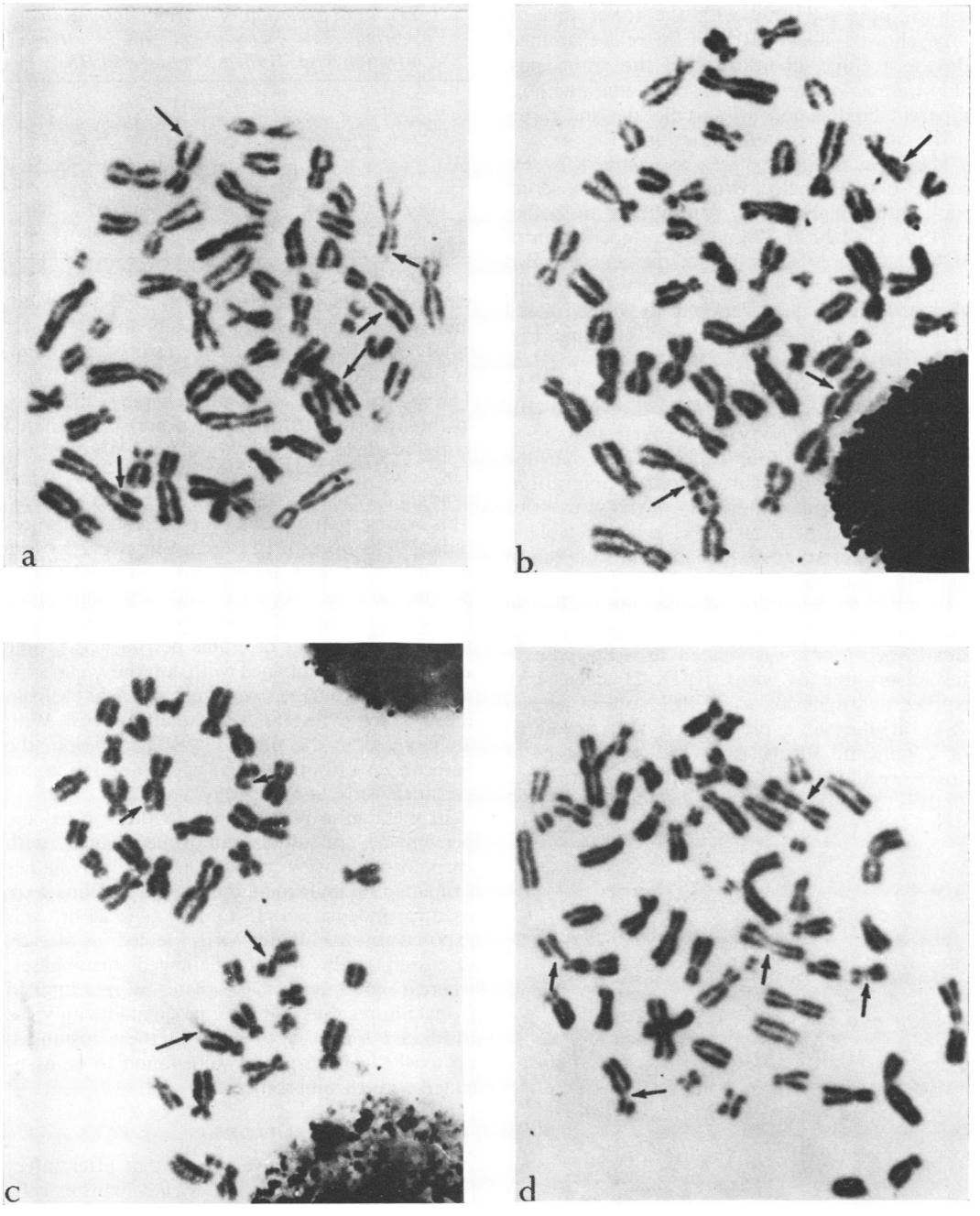

FIG. 2. *Metaphase plates 4 hr after infection with HSV and 1 hr after exposure to ^3H thymidine. Multiplicity of infections: 50 PFU/ml (a, b) and 5 PFU/ml (c, d). Chromosomal lesions are indicated by arrows.*

In experiments in which ^3H-thymidine-labeled HSV was used, cold thymidine was added to the medium during the 1-hr adsorption period (700 μg/ml) and during subsequent incubation (2 μg/ml). Table 1 shows the distribution of label in normal and abnormal metaphases 4 hr after infection of BHK-21 cultures at multiplicities of 10 and 100 PFU/cell. Nearly all of the abnormal metaphases revealed the presence of labeled viral DNA, whereas metaphases without evident

lesions, observed only with the lower dose of virus, showed label in 10% of the cells examined. On the average, about 18% of the grains possibly were associated with chromosomes, without apparent predilection for specific loci and rarely at the sites of chromosomal lesions (Fig. 4).

The virus specificity was ascertained by exposure of the labeled virus to a human serum pool, with a high titer of neutralizing antibodies to HSV, for 1 hr at 37 C prior to inoculation of BHK-21 cultures. Only 1% of the cells revealed label at 4 hr, and then only a very few grains, whereas control cells exposed to virus treated with FCS showed generally heavy labeling (Fig. 5).

Exposure of labeled virus preparations to increasing doses of UV irradiation revealed that the infectious property was inactivated at a rate approximately four times greater than the capacity to induce chromosomal aberrations (Fig. 6). The input multiplicity of the virus prior to irradiation was 50 PFU/cell; metaphase preparations were made 8 hr after inoculation of the BHK-21 cultures.

In order to determine whether the decline in the capacity of irradiated virus to induce chromosomal aberrations was related to a decrease in the adsorption of virus, BHK-21 cells were exposed in suspension to 50 PFU/cell of labeled HSV, or the same preparation was irradiated for 2 min and for 10 min. The same doses of virus were added to cell-free medium as zero-hour controls. Samples were removed from the cell-virus mixtures at intervals over a 2-hr period; they were centrifuged immediately and the supernatant fluids were assayed for radioactivity and, in the case of unirradiated virus, for infectivity. The results (Fig. 7) showed no striking differences in the adsorption of irradiated and unirradiated virus particles during 90 min of observation. Thereafter, a fraction of the radioactivity of irradiated virus was again released from the cells. This release may account for the fact that autoradiography of cultured cells at 6 hr after exposure to virus that had been irradiated for varying periods of time showed considerable differences in the percentages of labeled nuclei as well as in the average number of grains per nucleus (Table 2). The results obtained with labeled virus irradiated for 30 to 60 sec were indistinguishable from those noted with the unirradiated virus. With an increase in the dose of UV irradiation, the percentages of both labeled nuclei and the grains per nucleus declined steadily.

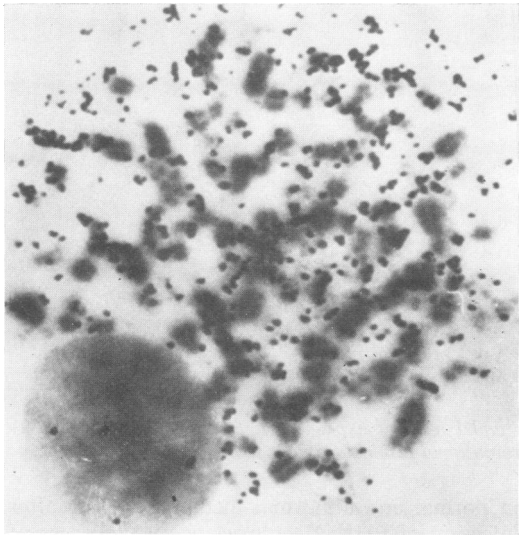

FIG. 3. *Metaphase plate 8 hr after infection with 5 PFU/cell and 1 hr after exposure to ^3H thymidine.*

TABLE 1. *Distribution of label in normal and abnormal metaphases prepared 4 hr after infection with ^3H thymidine-labeled HSV*

HSV (PFU/cell)	Per cent labeled nuclei	Normal metaphases		Abnormal metaphases	
		Total	Labeled	Total	Labeled
10	80	38	4	62	61
100	98	0	0	100	98

In metaphase preparations made at 6 hr, the percentages of silver grains associated with chromosomes were of similar orders, whether irradiated or unirradiated virus suspensions were used for inoculation (15 to 20%). At 24 hr, cells exposed to unirradiated virus yielded, as already discussed, only heavily damaged metaphases, whereas good metaphase plates were obtained from cultures that had been inoculated with virus irradiated for 4 to 10 min. In these instances, up to 48% of the grains were found to be associated with chromosomes (Fig. 8).

DISCUSSION

The experiments have shown that after infection of the BHK-21 line of Syrian hamster cells with HSV at high multiplicities, chromosomal aberrations became detectable at the earliest at 3 hr (9), and were prominent at 4 hr. A 1-hr pulse of ^3H-thymidine, applied just before the 4-hr harvest, generally yielded no evidence of DNA synthesis in affected metaphases. Thus, in contrast to a previous report (10), based upon work with a Chinese hamster cell line (which could account for the observed differences),

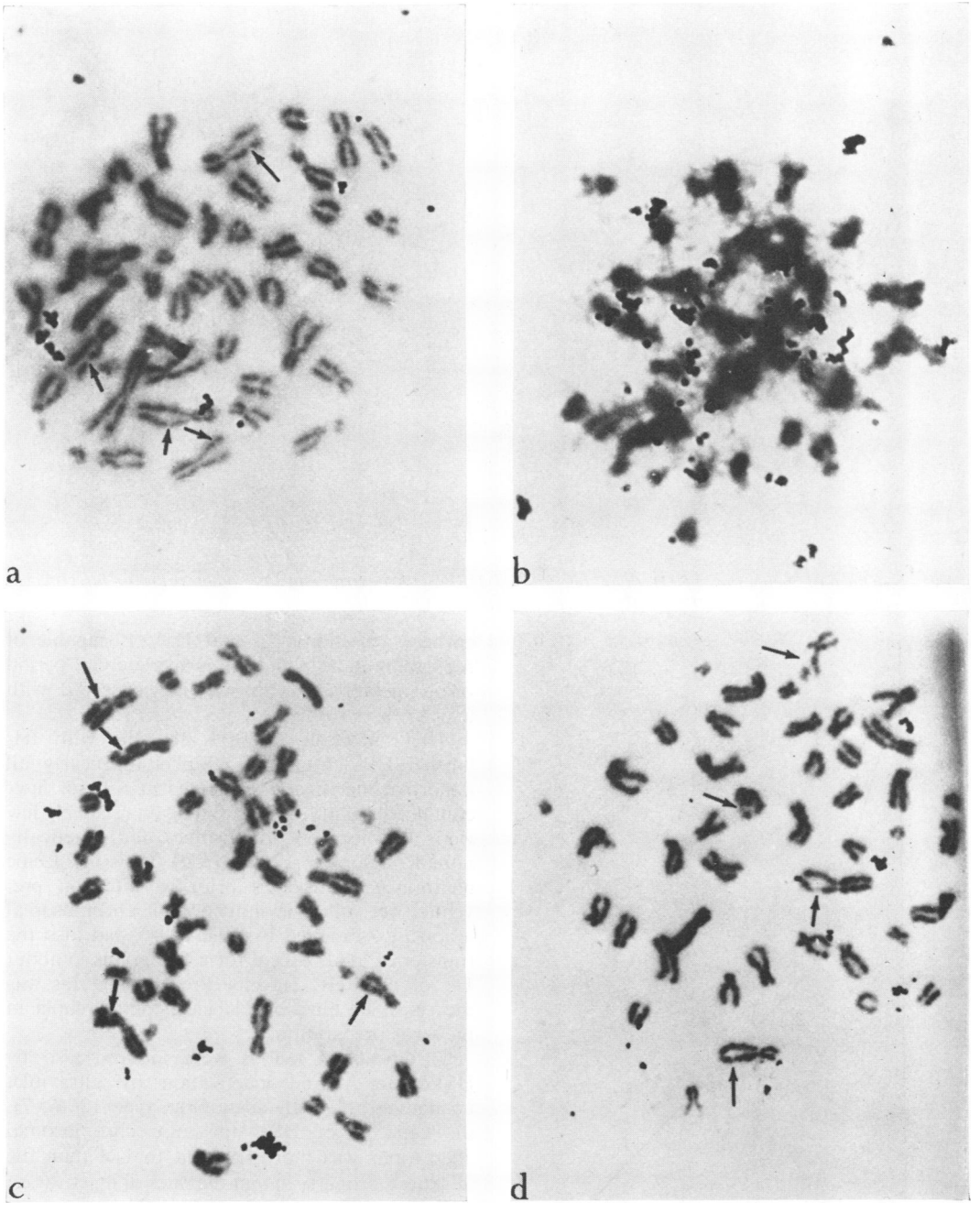

FIG. 4. *Metaphase plates 4 hr after infection with ^3H thymidine-labeled HSV (10 PFU/cell). Chromosomal lesions are indicated by arrows.*

induction of the chromosomal lesions was found to precede, and thus to be independent of, viral DNA replication.

These observations conformed to those made with adenovirus type 12 (6), but they differed in another aspect. After further incubation of HSV-infected cultures and application of 1-hr pulses of ^3H-thymidine before assay, most of the abnormal metaphases revealed the presence of extensive label. This result agrees with an

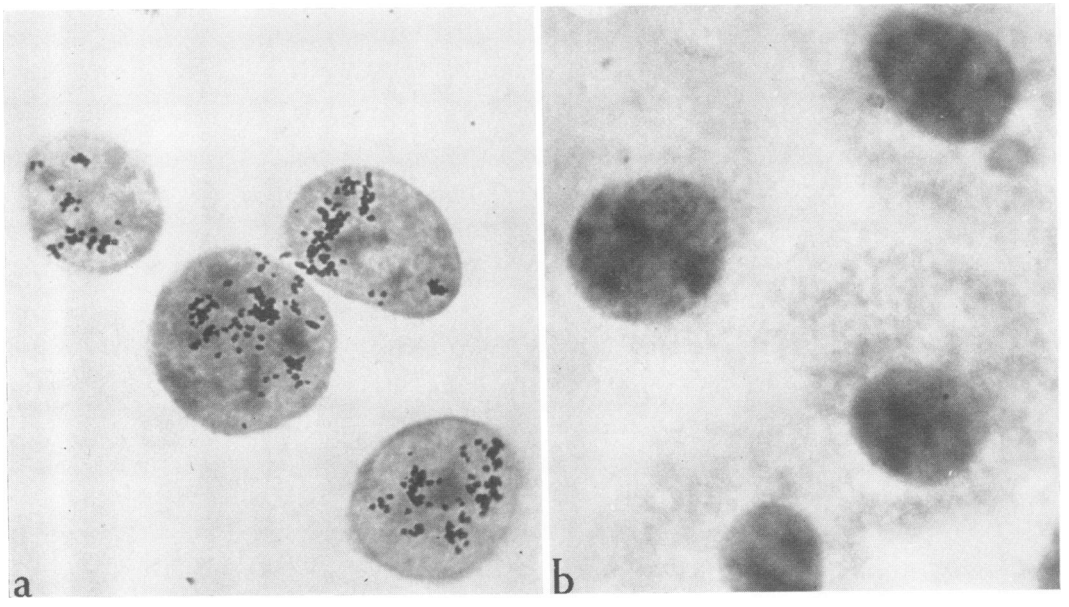

FIG. 5. *Effect of incubation of ^{3}H thymidine-labeled HSV with neutralizing antibodies prior to infection of BHK-21 cells. (a) Control (10 PFU/cell). (b) Neutralized virus (10 PFU/cell).*

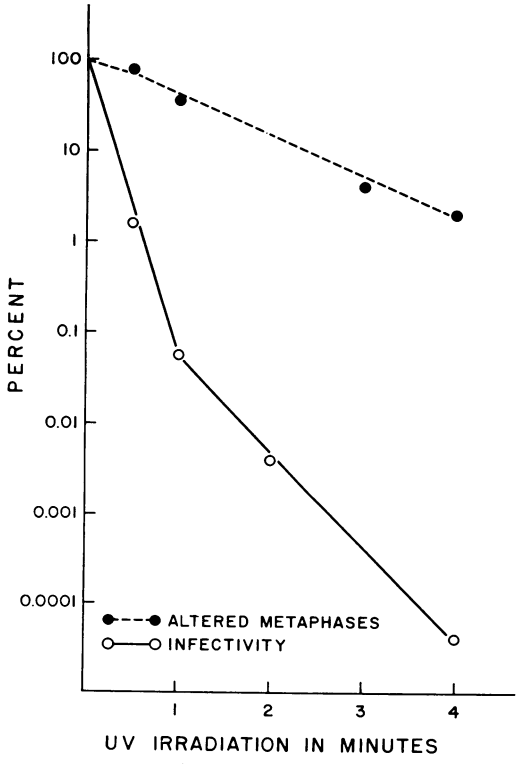

FIG. 6. *Effect of UV on the capacities of ^{3}H thymidine-labeled virus to infect BHK-21 cells and to induce chromosomal lesions. The nonirradiated virus (50 PFU/cell) induced chromosomal damage at 8 hr in 94% of the metaphases, which was taken as 100%.*

earlier suggestion (10) that HSV is capable of replicating in cells that are in the late G-2 period or in metaphase. This was not observed with adenoviruses (6, 13).

After infection of BHK-21 cells with ^{3}H-labeled HSV at high multiplicities, nearly all abnormal metaphases examined at 4 hr or later contained multiple grains, but again relatively few were attached to chromosomes and practically none to lesions. Exposure of the virus to specific neutralizing antibodies prior to infection prevented not only the induction of chromosomal lesions, as observed by others (14), but also the transfer of label, except for a few grains in about 1% of the BHK-21 cells. Presumably, this was the result of filterable, labeled contaminants in the virus preparation.

Chromosomal lesions were induced also by HSV after partial inactivation by ultraviolet irradiation. As with adenovirus type 12 (6, 7), the capacity of HSV to cause chromosomal aberrations was more resistant to UV than the infectious property in that the rates of inactivation differed by a factor of about four. The interpretation of these results was complicated, however, by the observation that after adsorption part of the radioactivity of heavily irradiated virus was again released from the cells. In line with this finding appears to be the fact that the percentages of labeled nuclei and the number of grains per nucleus decreased with an increase in the dose of UV irradiation of the ^{3}H-HSV suspension that was used for exposure of the cultures. Further-

more, after inoculation of heavily irradiated labeled virus, satisfactory metaphase preparations were still obtained after incubation for 24 hr. In these, nearly 50% of the silver grains present were found in association with chromosomes. The nature of these various changes in virus-host cell interactions is now being studied.

In conclusion, the data indicate that the chromosomal lesions induced by HSV are not a consequence of viral DNA replication or of its direct interaction with chromosomes. They suggest rather that the lesions might result from action of an early enzyme under control of the viral genome, as has been proposed also for similar effects caused by adenovirus type 12 (6, 7).

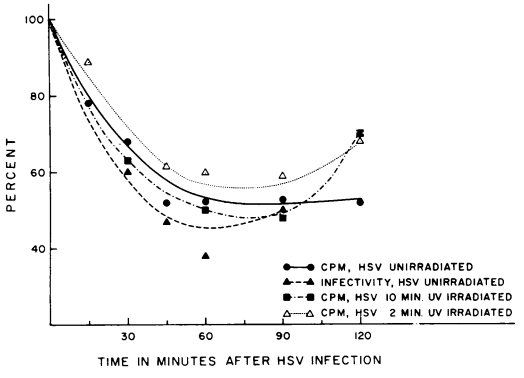

FIG. 7. *Adsorption of nonirradiated and irradiated ^{3}H-thymidine-labeled HSV onto BHK-21 cells in suspension.*

TABLE 2. *Effect of UV irradiation of ^{3}H-thymidine-labeled HSV on its capacity to enter the nucleus of BHK-21 cells*

Inoculum		Per cent labeled nuclei	Grains per nucleus[a]
HSV	UV (min)		
+	None	90	38
+	0.5	96	40
+	1	94	38
+	2	80	13
+	4	21	8
+	10	17	6
−	—	2	2

[a] Average of 100 nuclei were examined.

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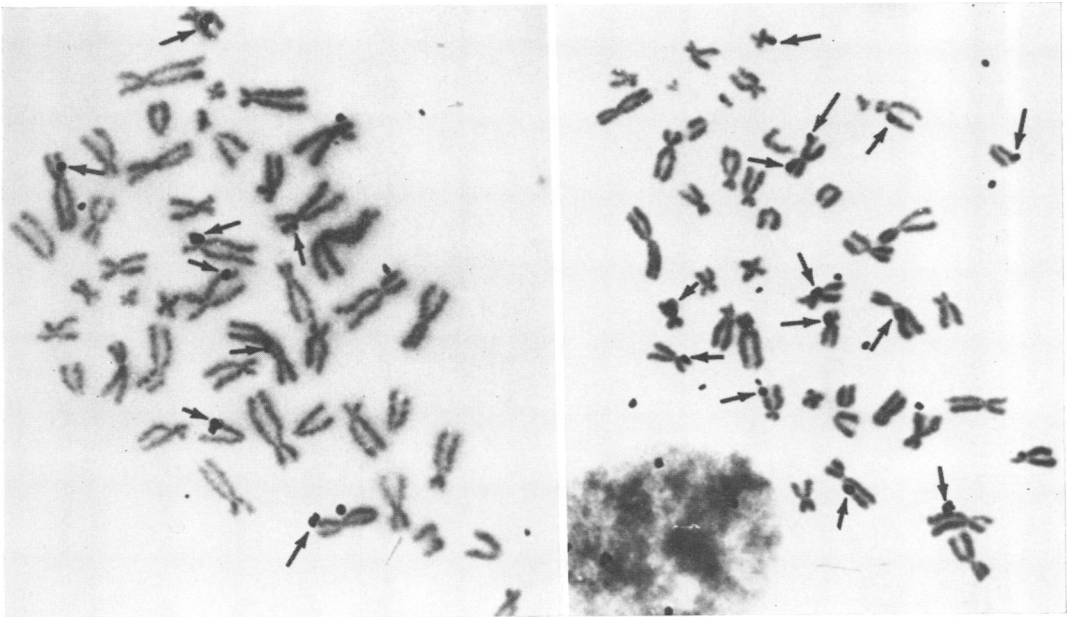

FIG. 8. *Metaphase plates 24 hr after exposure of BHK-21 cells to ^{3}H-thymidine-labeled HSV irradiated by UV light for 10 min. Most of the silver grains appear to be associated with chromosome (arrows).*

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