Cell-to-Cell Transmission of Herpes Simplex Virus in Primary Human Amnion Cells (36061)

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(Introduced by Wayburn S. Jeter)
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Several of the large DNA viruses spread throughout a monolayer cell culture by passing from previously infected cells to neighboring cells without releasing significant numbers of infectious particles into the culture medium (1–4). Herpes simplex forms clear plaques in monolayers of primary human amnion (5, 6). These lesions were shown by time lapse cinematography to be developed by the retraction of the cell sheet from the focus of infection. The infected cells form concentric rings or bands, which correspond roughly to the different generations of viral replication.

This report describes the application of tritiated thymidine radioautographic techniques to the study of the dynamics of virus-induced DNA synthesis within different bands of this microepidemic. In order to distinguish virus-induced DNA synthesis from normal cellular DNA synthesis, the latter was inhibited by exposing the cells to X-radiation prior to virus infection.

Materials and Methods. Virus. The strains of herpes simplex virus (HSV) used were primary human isolates. Three different isolates were employed during the course of the experiments, with each being used until it showed a tendency to form turbid plaques or giant cells.

Cells and medium. Primary human amnion cells were prepared by standard techniques as reported previously (7). Cells were cultivated on 9 × 22 mm coverslips in standard 16 × 150 mm tubes containing medium 199 supplemented with 20% human serum. When a confluent monolayer was formed, the fluid was changed to maintenance medium 199 with 5% calf serum. Stationary phase cells (5–10 days after trypsinization), were infected with 10–100 plaque forming units of HSV per cover slip to obtain discrete plaques.

Radioautography. The cultures were labeled in maintenance medium using Tdr^3H with a specific activity of 3.0 Ci/mmole. The cells were exposed for 1 hr to a concentration of 0.1 μCi/ml of Tdr^3H. In most experiments the coverslips were fixed after the hour of labeling, mounted cell side up on slides, dipped in Kodak Nuclear Tract Emulsion (NTB^3), and exposed for 14 days at 5°. They were stained by the methyl green pyronin method and inverted on clean slides for observation.

X-radiation. An X-ray unit operated at 250 kV and 15 mA with filtration through 1.01 mm of aluminum and 0.25 mm of copper was employed. The half-value layer was 1 mm of copper and the dose rate, measured in air, was 50 R/min. The total dose to which the cells on the coverglass was exposed was measured with a Baldwin dosimeter with the probe inserted into the glass tube. The cell monolayers were exposed to a single, continuous X-ray dose of approximately 5000 R.

Results. Uptake of Tdr^3H by stationary phase amnion cells: Primary human amnion cells were chosen for the study because it was believed that their cellular DNA synthesis would be minimal (8). These primary cells formed a confluent cell monolayer in 7 to 14 days; and, once cell-to-cell contact was established, the cells appeared to lie dormant for several weeks before they degenerated. A

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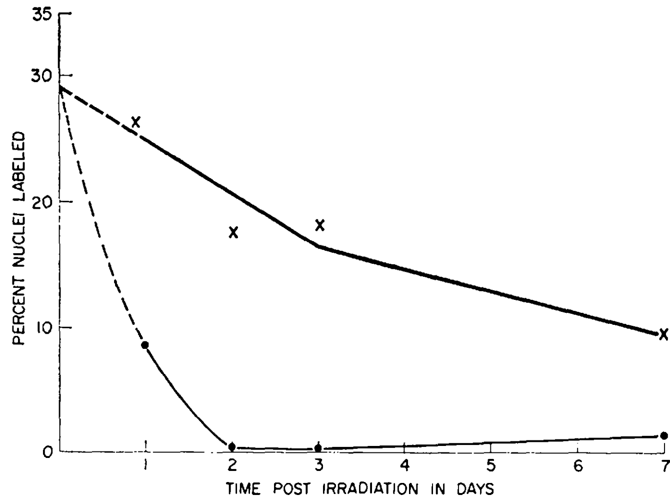

FIG. 1. The percentage of nuclei incorporation Tdr^3H in irradiated (●—); and nonirradiated (X—) amnion cells: The cells were irradiated at time 0 and were labeled at times indicated; the points represent the average of 4 experiments, (— —) follow unpaired points from the 4 experiments.

growth curve of such cells showed an initial loss during the traditional lag phase (3 days), a logarithmic phase (3 days), and a long stationary phase (21 days). The cell number in the stationary phase was less than twice the initial inoculum. Once the monolayer was formed, there was little cell division, as evidenced by the constancy of cell number and scarcity of mitotic figures. Although little cellular DNA synthesis would be expected in these cells, 30% of them incorporated Tdr^3H into their nuclei.

There may be three explanations for the incorporation of Tdr^3H into apparently resting cells: (i) the label is being incorporated into nuclear components other than DNA; (ii) the labeled compound is being exchanged for unlabeled thymidine in the cell pools; or (iii) the cells are dividing at a rate undetectable by the above criteria.

First, to determine whether the label was associated with the DNA or other nuclear fractions, infected and noninfected cells were labeled by the standard technique and fractionated by the Schmidt-Thannhauser-Schneider technique, cited in Ref. (9). The fractions were counted; and essentially all of the label was found in the DNA fraction, indicating that other nuclear fractions were not being labeled. Secondly, if the Tdr^3H replaces thymine or thymidine in the cell pools, this exchange should increase with time of exposure to Tdr^3H. The cells were labeled continuously for 6 hr with renewal of the label every 2 hr. Coverslips were fixed at 2 hr intervals and radioautographs were prepared. The percentage of cells incorporating the label did not increase over the period of the experiment. Grain counts demonstrated that the label in each nucleus in the S period did increase but the percentage of cells incorporating the label remained constant. This probably indicates that, under our conditions, only cells in the S period incorporate Tdr^3H. Thirdly, because there was no increase in cell number or obvious mitosis, it was difficult to determine generation time. To determine the doubling time, cells were labeled by standard techniques (0.1 μCi/ml of Tdr^3H for 1 hr); the label was removed and the cells were washed in an excess of unlabeled thymidine. These cells were maintained for 11 days in excess of cold thymidine and radioautographs were prepared at several periods. The labeled nuclei doubled in approximately 10 days, indicating the division of the cells that were in the S period at the time of labeling. This time may be somewhat long because the labeled thymidine itself may have caused mitotic delay (10) but it did

indicate that the cells were incorporating the label for cell division.

This background of cellular DNA synthesis did not necessarily invalidate the observations on cell-to-cell transmission of virus because the cells synthesizing DNA were randomly distributed throughout the culture, while those in viral DNA synthesis were restricted to focal areas. It did, however, obscure the viral DNA synthesis during the first 8 hr of virus infection when only the first cell in the subsequent plaque was infected. For this reason methods were sought to suppress cellular DNA synthesis.

Kaplan (11) and Minowada (12) reported that X-irradiation blocks cellular DNA synthesis but not DNA synthesis induced by a DNA virus. Such a procedure proved useful in the present study. The radioautographs of primary human amnion monolayers treated with 5000 R showed an almost linear decrease with time in the percentage of cells incorporating the label. As shown in Fig. 1, approximately 30% of the nonirradiated cells incorporated label into their nuclei. Within 2 days after radiation exposure, the percentage incorporation was well below 1% and remained at this low level throughout the experiment. Similar experiments with HeLa cells demonstrated almost complete cessation of DNA synthesis in 2 hr (Fig. 2). However, with HeLa cells there was a compensatory

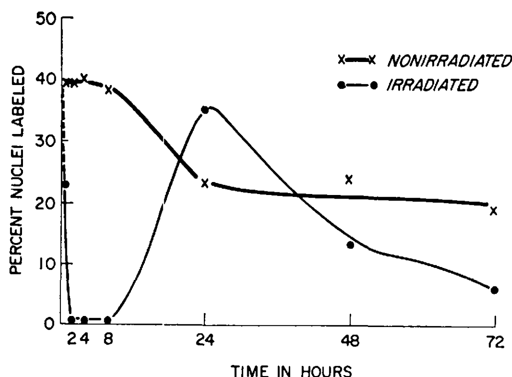

FIG. 2. The percentage of nuclei incorporating Tdr³H in irradiated (● —); and nonirradiated (× —) HeLa cells: The cells were radiated at time 0 and labeled at times indicated; The counts represent the percentage nuclei incorporating the label in one or two thousand cells.

TABLE I. Plaque Formation by HSV in Irradiated and Nonirradiated Amnion Cells.

Time of infection (days) after irradiation	No. of plaques/coverslip[a]	
	Irradiated[b]	Nonirradiated
1	155	152
2	157	39
3	99	41
7	47	29

[a] Plaques counted 24 hr postinfection.
[b] X-Ray doses, 5000 R.

rise in DNA synthesis which reached a peak at 24 hr. Such compensatory delays have been seen in other cell systems (13) and probably relate to mitotic delay and different sensitivities of the cells in different phases of the cell generation cycle. This compensatory wave has never been shown to occur with primary amnion cells.

This large dose of X-irradiation did not decrease the ability of the cells to support viral replication. As shown in Table I, the ability of irradiated cells to form plaques is at least as great as nonirradiated cells. This observation has been reported by others (11). Table I shows that irradiated cells are more sensitive to virus infection than nonirradiated cells. Because of the lower metabolic rate in the irradiated cells, it may be assumed that these cells are making less message and consequently the cell biosynthetic machinery is more readily available for viral message. Although X-irradiation does not interfere with virus replication, it essentially blocks cellular DNA synthesis. Consequently the DNA synthesis seen in an infected culture is almost exclusively viral induced DNA synthesis.

Tdr³H incorporation during the microepidemic. The amnion monolayers were irradiated 48 hr before infection and fixed at various times after infection. Radioautographs were prepared as described above. The development of the microepidemic is illustrated in Fig. 3. A focus of infection may be referred to as a microepidemic about 10 hr after infection (Fig. 3A). The plaque was then in the early second viral replication generation and the original cell was still in contact with the first band of infected cells. The

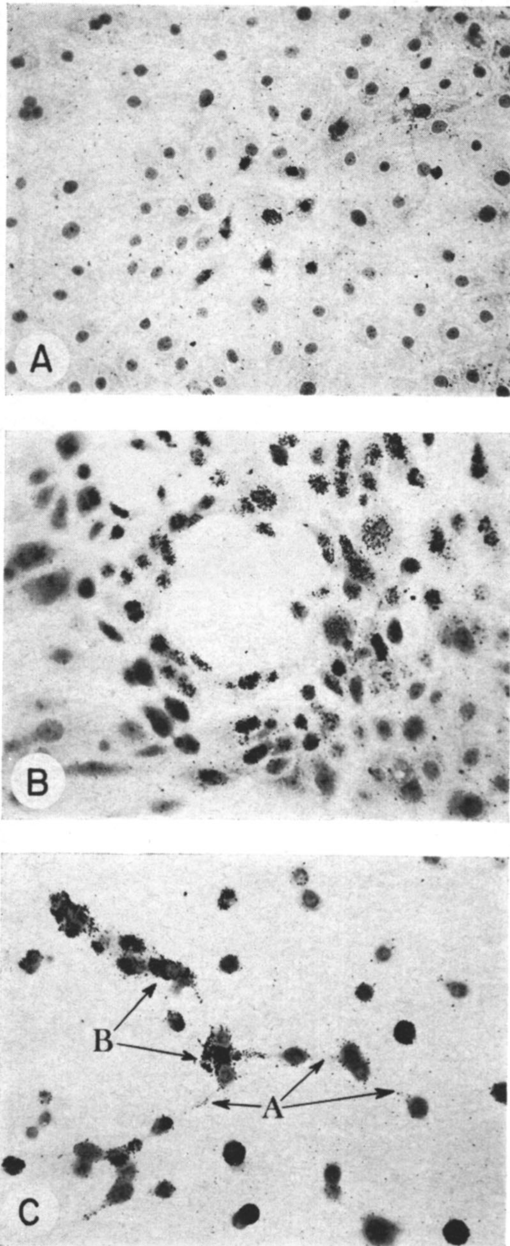

FIG. 3A. A radioautograph of a plaque 10 hr after infection with the first cell infected surrounded by seven cells in the second generation of viral replication. (B) A radioautograph of a herpes simplex plaque demonstrating the variation in intensity in the different bands of cells. (C) A plaque demonstrating the movement of the labeled DNA (A) through the intercellular bridges and (B) from the nucleus to the cytoplasm. The radioautograph was labeled 24 hr after infection and chased with cold thymidine for 24 hr before fixing.

third viral replication generation appeared between 16 and 18 hr. At 24 hr (Fig. 3B) the plaque retracted and demonstrated three concentric rings of DNA synthesis. The outermost bands (those infected for 2 to 4 hr) exhibit the most intense labeling.

We found the replication time of HSV in amnion cells to be between 6 and 8 hr. Roizman (14) and Kaplan (11), using other cell systems, report the same or slightly shorter replication times. However, a more meaningful value in the study of the microepidemic is the cell-to-cell transmission time. A transmission time of 8 to 10 hr may be calculated from the appearance of bands of infected cells in the plaques. This is an average time calculated from the time-lapse movies and the radioautographs fixed at different times. Because the virus moves by cell-to-cell transmission, only the cells in contact with the infected cells become infected. The time lapse cinematography demonstrates that a band of cells around the infected cells increase membrane and intercellular activity at about the same time. These cells then progress together to clear cytopathology. Representative time-lapse pictures are not shown here because it is their movement that is of interest and individual frames do not reproduce well. The autoradiographs are of better quality and demonstrate the same point.

The cell-to-cell transmission time may also be calculated by counting the number of cells synthesizing DNA during three replication generations. Irradiated coverslips were inoculated with approximately 100 plaque forming units of HSV/coverslip. The cultures were pulse labeled and radioautographs were prepared at 2 hr intervals for 24 hr. The radioautographs prepared from irradiated coverslips not inoculated with virus, demonstrated very few labeled nuclei (usually below 20 for a coverslip containing more than 500,000 cells). This background number was subtracted from the number of labeled nuclei seen in the inoculated cultures and the results are shown in Fig. 4. Experiments 54 and 56 identify two experiments in which the radioautographs labeled at different times were carefully analyzed. Figure 4 indicates that there are three plateaus in which the

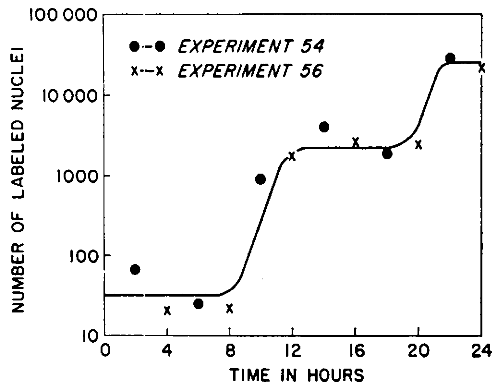

FIG. 4. The number of nuclei per coverslip incorporating Tdr^{3}H at various times after infection with 100 plaque forming units of HSV/coverslip: The coverslips were irradiated so there were very few labeled nuclei per coverslip in the noninfected cultures. For the early determinations, all of the labeled nuclei were counted. For the latter determinations, the percentage of nuclei incorporating Tdr^{3}H was multiplied by the total number of cells per coverslip. For the period between 10 and 20 hr, both techniques were used with a high degree of correlation.

number of labeled cells remain approximately constant. The duration of the plateaus is approximately 8–10 hr and then rises rapidly to the next level. This indicates that the cells are in DNA synthesis as early as 2 hr after infection; and between 8 and 10 hr, the second generation of cells is infected and induces DNA synthesis in the second generation of cells.

The determination of the fate of the label, which was incorporated under the influence of HSV, was accomplished by modifying the usual labeling techniques. The amnion cells were infected with HSV for a period of 24 hr, then exposed to Tdr^{3}H for 1 hr as in the normal procedure. Then, the cells were washed and placed in a medium containing an excess of unlabeled thymidine for an additional 24 hr of incubation. They were then washed and fixed; and radioautographs were prepared in the usual manner.

As illustrated in Fig. 3C, the amnion cell monolayer, which was infected 48 hr before fixation, had retracted leaving only a few cells with long intercellular bridges. The label, which represents viral induced DNA synthesized 24 hr after infection, had migrated from its site of synthesis (the nucleus) into the cytoplasm and intercellular bridges. The appearance of the label in the intercellular bridges is in agreement with the previously reported data by Hoggan *et al.* (15) using fluorescent herpes antibody to detect HSV within the intercellular bridges of infected cells. The fluorescent antibody studies have been repeated in this laboratory using the HSV primary human amnion cell system. Specific HSV antigen was found in the intercellular bridges.

Discussion. The microepidemic is of interest because it may explain the mode of infection and persistence of HSV, as well as other large DNA viruses that replicate in the nucleus. This system also allows the observation of a single plaque forming unit during several replication generations. Tdr^{3}H radioautographic, time-lapse cinematography, and fluorescent antibody techniques (15) have been employed to study this interesting phenomenon.

Time-lapse cinematography is perhaps the best technique to observe the interaction of cells during the formation of a microepidemic. Unfortunately, it is difficult to describe these complicated movements in words or illustrate the dramatic interactions in pictures. However, these movies did show three important characteristics of plaque formation in this system. First, the plaque was formed by retraction of the cell sheet. Second, only cells in direct contact with infected cells became infected. Third, the sequence of infection was preserved as the cell sheet retracted. This process results in a microepidemic in which all of the cells, which have been infected, are present and lined up in rings around the plaque. Each band or ring of cells represents a viral replication generation in that this band (which is one cell thick) must be infected and then replicate virus for the infection of the next more peripheral band of cells. Fluorescent antibody studies confirmed the presence of specific viral protein in these cells (15).

By means of Tdr^{3}H radioautographs the development of a microepidemic may be followed by the induction of DNA synthesis in irradiated cells. The irradiated primary cell,

under the conditions of the experiment, is incapable of initiating DNA synthesis unless stimulated by the virus. This technique allows the observation of many plaques on a coverslip at different stages of plaque development. The cell-to-cell generation time was determined by observing the appearance of the labeled rings of cells in many plaques and compared to the time of appearance of rings of cells in individual plaques in time-lapse cinematography. A third technique was used to determine the cell-to-cell transmission time. Figure 4 shows that the number of labeled nuclei increases dramatically at 8 to 10 hr intervals indicating the infection of the next ring or band of cells.

The cell-to-cell transmission time found in these studies is not the same as the viral replication time determined by the usual 1 step growth curve experiments. In these studies the cells are infected and ruptured at various times after infection and assayed for infectious material. Roizman (14) found the virus replication time to be between 5 and 6 hr. Using similar techniques, we have confirmed this in our system. The cell-to-cell generation time includes the replication as well as the transmission times.

The microepidemic appears to develop largely by transmission of the virus through intercellular bridges. Both labeled DNA and specific HSV protein may be found in these intercellular bridges.

The investigation of the microepidemic raises several points concerning the basic biology of the virus infection that are not resolved by this study. Dulbecco *et al.* (16) and several other investigators have shown that DNA viruses can induce cellular DNA synthesis in contact-inhibited cells. The DNA seen in herpes-infected cells is probably largely viral because, as shown in Fig. 3C, the labeled DNA migrates in the cell as the virus would be expected to migrate. Cellular DNA would be expected to remain in the nucleus rather than move into the cytoplasm and intercellular bridges. However, cellular DNA synthesis may be induced under some circumstances early in the infection.

Another interesting point would be to determine whether the entire viral genome is necessary to initiate DNA synthesis in irradiated cells. Defendi and Jensen (17) and Latarjet *et al.* (18) have shown the property of tumor induction in small oncogenic DNA viruses to be more resistant to radiation than the property of virus infectivity. This is interpreted to mean that less of the viral genome must be read by the cell for tumor induction than for infectivity. This may also be true for stimulation of DNA synthesis in irradiated cells. Studies are now in progress to determine whether HSV inactivated by irradiation and heat are able to stimulate DNA synthesis in irradiated cells. The results of this investigation may indicate whether the viral information necessary to stimulate DNA synthesis, like the information for transformation, can be shown in less than the whole viral genome. This information, although important, will probably add little to the study of the microepidemic.

Summary. The microepidemic in primary human amnion cells infected with herpes simplex virus was studied by tritiated thymidine (Tdr^3H) autoradiography, time-lapse cinematography, and fluorescent antibody techniques. The plaque is formed by retraction of the cell sheet and the sequence of infection is preserved in rings of cells around the plaque. The progress of the plaque may be followed in irradiated cultures in which essentially all of the DNA synthesis is virus induced. The cell-to-cell transmission as opposed to the cell generation time was 8–10 hr as determined by three different techniques. The presence of labeled DNA and specific HSV protein in the intercellular bridges indicates the means by which the virus spreads in a culture.

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