Cellular Deoxyribonucleic Acid Synthesis and Loss of Contact Inhibition in Irradiated and Contact-Inhibited Cell Cultures Infected with Fibroma Virus¹

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Cultural changes that follow infection of rabbit kidney cells with fibroma virus were studied. Characteristic alterations of cell morphology and development of multilayered piles and cords of cells were found to occur in infected cultures in which cell division was blocked by gamma radiation or by cell crowding and serum deprivation, thus indicating no dependence upon cell division. Fibroma virus infection did not remove blocks to cell division, but it did exert distinct effects upon nuclear deoxyribonucleic acid synthesis in cells blocked by radiation or cell crowding. Use of tritium-labeled thymidine and autoradiography demonstrated that after infection initial inhibition of nuclear incorporation was followed by sharply increased nuclear labeling at a time that coincided with beginning alterations of cell morphology and development of cell piling.

In a previous communication (8), it was demonstrated that infection of primary and serially cultured domestic rabbit kidney cells with the Patuxent strain of rabbit fibroma virus resulted in altered cell morphology and loss of contact inhibition followed by proliferation of the infected cells in an abnormal growth pattern. This response has some similarities to *in vitro* transformation or conversion by other oncogenic viruses. However, proliferation of cells after infection with fibroma virus has not been found under all circumstances. Israeli and Sachs (10) observed a higher mitotic frequency after infection of secondary rabbit testes cells, but not in rabbit or rat embryo cells. The increased mitotic frequency coincided with the time of pock formation. More recently, studies of Israeli utilizing mitotic inhibitors (9) suggested that the mechanism of fibroma virus-induced pock formation on secondary and cloned rabbit embryo and testes monolayers was one of cell aggregation and not cell multiplication. Verna (20) described a diverse response to fibroma virus in three established rabbit cell lines ranging from cell degeneration to pock formation in the absence of any detectable

cytopathic effect; whereas Kato et al. (12) emphasized the lack of cell division in human FL cells infected with fibroma virus. Hinze and Walker (*unpublished data*) have found strains of fibroma virus that rapidly destroy cells with little evidence of cell multiplication.

These varied results suggest that the response to fibroma virus depends upon the host cell and upon the nutritional and environmental conditions of the culture but, in addition, allow the hypothesis that the previously described cell alteration and cell proliferation are independent events subject to different controls in infected cultures. The experiments described here were designed to test this hypothesis by utilizing non-dividing cell populations. The results support the view that infection of rabbit cells by fibroma virus can lead to alteration of cell morphology and loss of contact inhibition in the absence of cell division. They indicate, in addition, that fibroma virus can affect cellular controls to the degree that a burst of nuclear deoxyribonucleic acid (DNA) synthesis occurs in cell cultures previously under nuclear inhibition as a result of gamma irradiation or cell contact.

MATERIALS AND METHODS

Cells. Domestic rabbit kidney (DRK) cells, a strain of epithelial cells of New Zealand white rabbit origin (8), were used between the 20th and 35th subcultures.

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Stock cultures were grown in 250-ml plastic tissue culture flasks (Falcon Plastics, Los Angeles, Calif.) in medium 199 with additions of 0.25% lactalbumin hydrolysate, 100 units of penicillin per ml, 100 μ g of streptomycin per ml, and 10% commercial calf serum. The pH was adjusted to 7.2 to 7.4 with sodium bicarbonate. Cultures were subcultured at weekly intervals.

Virus. The Patuxent strain of Shope fibroma virus (obtained from the American Type Culture Collection) was prepared as stock virus by inoculating DRK cells in medium 199 and 10% calf serum and harvesting after 60 hr of incubation at 35 C. The cell sheet was removed by freezing and thawing, and the suspension was sonically treated to release cell-bound virus. Cell debris was removed by light centrifugation, and the supernatant fluid was stored at -70 C. The virus titer was determined by means of an *in vitro* plaque assay as previously described (16). Virus preparations contained between 5×10^6 and 10^7 plaque-forming units (PFU) per ml.

Preparation of cell cultures. Cultures for experimentation were prepared as previously described (8). Cells were resuspended in medium 199 plus 10% calf serum, and 2-ml amounts were dispensed into flat-bottom shell vials containing cover slips (Kimble no. 1, 18 mm). Cells were allowed to attach to cover slips and were incubated for 18 to 24 hr at 37 C before infection. All cultures, unless otherwise specified, were refed at 24-hr intervals with 2 ml of medium (pH 7.2 to 7.4) to maintain conditions favorable for proliferation of infected cells.

Irradiation of cultures. Monolayer cultures of DRK cells in 250-ml plastic flasks containing 10 ml of medium were exposed to 5,000 r of gamma radiation from a caesium-137 radiation unit (Caesatron Model C, Atomic Energy of Canada, Ltd.). The cultures received a dose of 95.6 r/min at a distance of 25 cm from the source. Immediately after irradiation, cells were dispersed with trypsin and were seeded into shell vials on cover slips. After 72 hr at 37 C, the cultures were used in experiments.

Infection of cultures. Cultures were inoculated with a virus input of 2 PFU/cell. Virus was added in 0.2 ml of medium and allowed to adsorb for 3 hr at 37 C. After adsorption, 2 ml of medium was added, and the cultures were incubated at 37 C for 18 to 24 hr. Thereafter, the medium was changed at 24-hr intervals.

Preparation of cultures for staining. Cell sheets on coverslips were washed twice with phosphate-buffered saline (PBS), fixed in 1 ml of Bouin fixative for at least 5 min, and stored in 2 ml of 70% ethyl alcohol for 24 hr before staining with Harris hematoxylin and eosin.

Autoradiography. Tritiated thymidine (^3H -TdR) with a specific activity of 6.7 c/mmole was obtained from New England Nuclear Corp., Boston, Mass. It was added to cultures in medium 199. All pulse experiments used 0.5 ml of labeled medium per vial. Continuous labeling experiments were done with ^3H -TdR in 2 ml of medium which was changed at 48-hr intervals. After treatment of cultures with ^3H -TdR, they were washed twice with saline and immediately fixed in a solution of 70% ethyl alcohol, neutral Formalin (40% Formalin saturated with MgCO_3),

and glacial acetic acid (20:2:1) for 5 min. They were then mounted cell side up on glass slides. The dried slides were dipped in Kodak Nuclear Track Emulsion (Eastman Kodak Co., Rochester, N.Y.), sealed in boxes, and stored at -20 C for 4 days. The autoradiographs were then developed, fixed, and stained with hematoxylin and eosin. Cover slips were placed on top of the autoradiographs, and the percentage of cells with label in the cytoplasm or nucleus was determined by examining and counting 1,000 cells at a magnification of 440. Each sample number represents the average of two autoradiographs.

RESULTS

Response of irradiated DRK cultures to infection by fibroma virus. An experiment was designed to test for cell replication as a prerequisite for expression of altered cell morphology and loss of contact inhibition following infection by fibroma virus. Irradiation was used to impose a permanent arrest on cell division while permitting infection in a favorable nutritional environment. Cultures of DRK cells were exposed to 5,000 r of ionizing radiation. Immediately after irradiation, cells were dispersed with trypsin and seeded into shell vials with cover slips in 2 ml of medium at a concentration of 2×10^5 cells/vial. Cultures were incubated at 37 C for 3 days with daily medium change. After this time, half of the cultures were inoculated with 2 PFU of fibroma virus/cell in 0.2 ml of medium. The remaining cultures served as uninfected irradiated controls. After virus adsorption, medium was added and, 12 to 18 hr later, the cultures were refed with fresh medium. One half of a group of nonirradiated cultures containing 2×10^5 cells/vial was inoculated as above and the other half was used for nonirradiated uninfected controls. All cultures were refed daily with 2 ml of medium. Duplicate samples for cell counts and virus assays were taken at 24-hr intervals.

Infection of nonirradiated growing cultures (Fig. 1) resulted in an initial decline in cell numbers followed by a proliferative response. Two days after infection, the cell population was reduced by approximately 45%, after which time the surviving cells began to proliferate, reaching maximal numbers at 7 to 8 days after infection. This response was similar to that described in an earlier study (8). In contrast, lethally irradiated cultures demonstrated a slow but steady decline in cell numbers, such that at 8 days after irradiation the cell population was reduced by approximately one-half. Infection of irradiated cultures did not cause either an initial rapid decline or a secondary increase in cells in the cultures, and cell numbers paralleled the curve for irradiated control cultures.

Maximal cell-associated virus titer in irradiated and nonirradiated cultures was observed at 48 hr

FIG. 1. Effect of fibroma virus on the growth of irradiated and nonirradiated DRK cells. Cultures were inoculated with 2 PFU/cell on day 0. Irradiated cultures were exposed to 5,000 r 3 days prior to infection. Each point represents the average of the total number of cells in each of two tissue culture vials.

after infection, with a parallel decline in both systems thereafter (Fig. 2). Virus titers in non-irradiated cultures declined after the 2nd day of infection at approximately the same time that increase in cell numbers could first be demonstrated in infected cultures. This was a decrease in both virus per cell and total virus per culture. The decline in virus level was found in a previous study (8) and appeared to be related to the beginning of multiplication of infected cells. However, the present study demonstrated that, although cell division is blocked by irradiation, the decline in virus titer starts at the same time and parallels that seen in multiplying cells.

Even though cell counts provided no evidence for cell multiplication in infected irradiated cultures, morphological changes observed were similar to those seen in multiplying nonirradiated cultures after infection by fibroma virus. Little change was evident up to 48 hr after infection, other than cell enlargement and vacuolization. Three days after infection, both irradiated and nonirradiated cultures displayed characteristic cell elongation and loss of contact inhibition, as manifested by disorientation and multilayering of cells. Six days after infection, foci of piled-up cells were marked in both instances (Fig. 3a and b). Cell sheets around the foci in irradiated cultures were relatively thin and incomplete. Also, the cell density of the foci in irradiated cultures was characteristically less than in the foci of nonirradi-

ated cultures, supporting the observation that nonirradiated cells were able to proliferate after the 2nd day of infection, whereas irradiated cultures were not. Irradiated control cells increased in size and formed some polykaryocytes after several days. No other changes were observed up to 12 days after seeding.

DNA synthesis in irradiated DRK cells infected with fibroma virus. Since fibroma virus multiplies only in the cytoplasm (11), autoradiography allowed a comparison of viral and cellular DNA synthesis in irradiated and nonirradiated cultures. At 12- or 24-hr intervals, cultures were given 2-hr pulses at 37 C with 0.5 ml of medium containing 0.1 μ Ci of ³H-TdR/ml and immediately washed with saline and fixed for autoradiography.

Cytoplasmic labeling, representing viral DNA synthesis, was at a maximum at the 12-hr pulse in both irradiated and nonirradiated cultures. At that time, between 50 and 60% of the cells in both types of culture demonstrated viral DNA synthesis in the cell cytoplasm. At 24 hr, label was taken up in less than 5% of cells. At 36 hr, cytoplasmic labeling was about 3% in both types of culture and it did not exceed 2% at all subsequent test times. At all test times it was evident in many

FIG. 2. Multiplication of fibroma virus in irradiated and nonirradiated DRK cells. Cultures were inoculated with 2 PFU/cell on day 0. Irradiated cultures were exposed to 5,000 r 3 days prior to infection. Cells were trypsinized, counted, sedimented by centrifugation, and sonically treated to release cell-associated virus before assay. Each point represents the average results of two tissue culture vials.

FIG. 3. Morphological and cultural changes after infection of irradiated and nonirradiated DRK cultures with fibroma virus. Cultures were inoculated with 2 PFU/cell 1 day after seeding, which was 3 days after exposure to 5,000 r for the irradiated cultures. (A) Nonirradiated, 6 days after infection; (B) irradiated, 6 days after infection. $\times 100$.

cells that label grains were overlying viral inclusion bodies.

Uninfected exponentially dividing cultures showed (Fig. 4) a large fraction of cells with nuclear labeling at the time of the first three pulses; however, as the cultures increased in cell number and division was inhibited by cell crowding, the number of cells incorporating nuclear label declined rapidly. Infection of exponentially dividing cultures caused an almost complete inhibition of nuclear labeling at 24 and 36 hr after introduction of the virus, as was previously observed (Hinze, H. and D. Walker, *Bacteriol. Proc.*, p. 136, 1966). By 48 hr, however, nuclear labeling was again evident, and the number of nuclei accumulating label increased steadily to a maximum on the 4th day after infection, at which time it was well above the highest value observed in uninfected control cultures. A decline in the

number of nuclei incorporating label occurred after the 4th day. Irradiation of cells with 5,000 r reduced the number of cells showing ^3H -TdR incorporation into the nucleus during a 2-hr pulse to approximately 20% of exponentially growing controls, and the fraction of irradiated cells incorporating ^3H -TdR declined slowly throughout the experiment. However, when irradiated cells were infected with fibroma virus, the same general pattern of labeling evolved as was seen in infected nonirradiated cultures, although fewer cells participated. There was an inhibition of nuclear labeling at 24 hr after infection, which was released at 48 hr; thereafter, an increased number of nuclei took up label as compared to controls. The fraction of cells incorporating nuclear label reached a maximum on the 4th day after infection, at which time it was approximately three times that of irradiated controls. The elevated frequency of labeled nuclei in infected cultures

FIG. 4. Cellular DNA synthesis in irradiated and nonirradiated DRK cultures after infection with 2 PFU of fibroma virus/cell. Irradiated cultures were exposed to 5,000 r 3 days prior to infection. ^3H -TdR incorporation into the nuclei measured by means of autoradiography. Cultures were pulsed at intervals shown for 2 hr with $0.1 \mu\text{C}$ of ^3H -TdR/ml in 0.5 ml of medium at 37 C. Each point represents the average percentage of labeled nuclei in 1,000 cells counted in each of two autoradiographs at a magnification of 440. Each curve represents the average of two separate counts of each sample.

persisted for 1 to 2 days and then began to decline toward control values.

This experiment was repeated on several separate occasions, and results were always similar to those presented. The ranges of counts in replicate counting of autoradiographs are shown in Fig. 4. They were greatest in proliferating infected cultures because of the formation of dense foci of multilayered cells that began to appear after the 3rd day of infection and made scoring of individual cells more difficult.

Tests for cell division in irradiated cultures. Although there was no increase in numbers of cells in cultures of DRK cells that had been exposed to 5,000 r and then infected with fibroma virus, the possibility remained that multiplication of a portion of the cell population did take place but was hidden by concurrent death and lysis of another fraction of the cell population. Such a fraction of multiplying cells could arise from a radiation-resistant minority or from an effect of fibroma virus that might allow some cells to escape the block of multiplication usually imposed by radiation. The possibility that a fraction of the population could be multiplying was made more reasonable by the observation that fibroma virus infection of irradiated cultures was followed by a surge of nuclear DNA synthesis. Several experiments were conducted, therefore, that were aimed at revealing repeated divisions in any fraction of the cell population.

Cell cloning experiments revealed that not more than 1 in 10^5 cells from irradiated but uninfected cultures was capable of colony formation. No cells capable of colony formation could be found among many millions of irradiated cells after infection with fibroma virus. This suggested that any capacity for multiplication to be found in irradiated cells after fibroma virus infection was limited to a small number of divisions.

Another experiment followed the accumulation of labeled nuclei in irradiated and unirradiated

cultures. It was reasoned that, if a fraction of the cell population were capable of unlimited divisions, continuous exposure to ^3H -TdR should result in progressively increasing numbers of labeled nuclei as a function of time. Irradiated and nonirradiated cells were infected with a virus input of 2 PFU/cell as in the previous experiment. After a 3-hr adsorption period at 37 C, cultures were fed with 1 ml of medium containing ^3H -TdR ($0.05 \mu\text{C}/\text{ml}$). Uninfected irradiated and nonirradiated cultures were treated in the same way. All cultures were refed 12 hr later with the same medium and every 48 hr thereafter. Duplicate samples were taken at 24-hr intervals for autoradiography.

The data given in Table 1 represent the averaged results of two experiments. It was observed that in both control and infected nonirradiated cultures virtually 100% of the cells were labeled after several days of exposure to ^3H -TdR. This was as expected in view of the increase in cell numbers seen in such cultures in previous experiments. On the other hand, in control and infected irradiated cultures, the number of cells with labeled nuclei plateaued at 50%. Cessation of incorporation at the 50% level would not be expected if the irradiated cells or a portion of these were capable of unlimited division, since the cultures were not confluent at this time but had actually decreased in number (Fig. 1). The total number of cells eventually incorporating label was approximately the same for infected and uninfected irradiated cultures, although the rate of accumulation of labeled nuclei was quite different and appeared to reflect the different patterns of nuclear synthetic activity shown in Fig. 4. In this experiment, 70 to 80% of infected cells (both irradiated and unirradiated) had one or more cytoplasmic centers of viral DNA synthesis at 24 and 48 hr after infection.

Response of contact-inhibited cells to fibroma virus infection. Several experiments demonstrated

TABLE 1. Accumulation of labeled nuclei in uninfected and infected cell cultures after irradiation

Cell culture	Cumulative percentage of labeled nuclei ^a (days after addition of labeled thymidine) ^b							
	1	2	3	4	5	6	7	8
Nonirradiated, uninfected	84.3	97.2	99.6	100	100	100		
Nonirradiated, infected ^c	15	21.1	64.1	76.0	~100	~100	~100	
Irradiated, ^d uninfected	22.1	32.0	33.4	43.9	48.1	47.9	46.1	
Irradiated, infected	8.0	9.2	19.0	26.1	~28.0	~50	~50	~50

^a Average percentage of cells with nuclear label in 1,000 cells counted in each of two autoradiographs.

^b Cultures were exposed to $0.05 \mu\text{C}$ of ^3H -TdR/ml of medium throughout the experiment.

^c Cells inoculated with 2 PFU of fibroma virus 3 hr before addition of ^3H -TdR.

^d Cells exposed to 5,000 r of gamma irradiation 3 days prior to addition of ^3H -TdR.

that crowding of cells or removal of serum from the medium stopped the multiplication of DRK cells and that the addition of fibroma virus to such cultures (2 PFU/cell) did not result in increased numbers of cells. However, although fibroma virus infection did not appear to overcome the blocks to cell division imposed by contact inhibition or deficient medium, the cells developed characteristic morphological alterations and moved into piles and cords that could be recognized 3 days after infection.

Autoradiography revealed that fibroma virus infection had a marked influence on nuclear incorporation in contact-inhibited cultures even though there was no evidence for cell division. DRK cells were seeded into shell vials at a concentration of 2×10^5 cells/vial and grown to confluency. One day after becoming confluent, one group of cultures was inoculated with 2 PFU/cell in the usual manner; this group and a control group of uninoculated cultures were maintained in medium containing 10% serum. Serum was removed from the medium of another group of cultures, and 1 day later half of the cultures were inoculated with virus and the other half served as uninoculated controls. These cultures were refed every 24 hr. Duplicate samples were removed from each of the four sets of cultures every 12 hr for staining and autoradiography. The results (Fig. 5) were found to be reproducible in a second experiment. Infected cultures with or without serum demonstrated a virus-induced inhibition of nuclear ^3H -TdR incorporation at 24 hr after infection, with resumption of nuclear labeling at 48 hr. At 72 hr after infection in serum-free medium, approximately 12% of the cells incorporated ^3H -TdR in a 2-hr pulse as compared with approximately 1% in uninfected serum-free cultures. A similar increase in nuclear DNA synthesis was seen at 72 hr in those infected cultures held in medium containing serum. Microscopic studies revealed that confluent cultures no longer displayed contact inhibition at 72 hr after infection, as demonstrated by random arrangement and piling up of cells. This coincided with the time of increased uptake of label into the nuclei.

DISCUSSION

After infection with fibroma virus, growing cultures of DRK cells undergo characteristic changes in cell morphology and then multiply in an abnormal pattern that indicates lost responsiveness to contact inhibition. The experiments reported here demonstrate that the changes in cell morphology and the abnormal cultural pattern are not dependent upon cell division. Both gamma radiation and cell crowding were effective in stopping multiplication of DRK cells, and fi-

FIG. 5. Cellular DNA synthesis in confluent cultures with and without serum after infection with 2 PFU of fibroma virus/cell. Serum was removed from cultures 1 day before infection, and these cultures were refed with serum-free medium thereafter. Cultures were pulsed at the intervals shown for 2 hr with 0.5 ml of medium containing $0.1 \mu\text{Ci}$ of ^3H -TdR/ml. Each point represents the average percentage of labeled nuclei in 1,000 cells counted in each of two autoradiographs at a magnification of 440.

broma virus infection did not relieve the blocks to cell division. Nonetheless, infected cells went through the characteristic change in morphology and formed multilayered piles and cords. These observations fit with those of Israeli (9) and Israeli and Sachs (10) on pock formation by fibroma virus in rabbit cell monolayers. Somewhat similar dissociation of cell movement and cell division has been demonstrated in human fibroblasts infected by Rous sarcoma virus (14).

Although fibroma virus infection does not remove the block to cell division that is imposed by either radiation or cell crowding, it does exert a distinct influence on cellular DNA synthesis in irradiated cells. Infection appears to initiate in irradiated or contact-inhibited cultures the same sequence of nuclear synthesis that is seen in growing cultures after fibroma virus infection. Pulse-labeling experiments demonstrated an initial inhibition of nuclear incorporation, followed by a sharp increase in nuclear labeling that reached levels considerably above those of control cultures. The surge in nuclear incorporation after infection was particularly prominent in cultures

blocked by a combination of contact inhibition and serum deprivation. This suggests that nuclear synthesis has come under some form of viral control or that nuclear synthesis is markedly influenced by viral effects on other parts of the cell. It appears that, although fibroma virus has some capacity for inhibition of nuclear function, this is temporary, and a second virus-induced effect occurs that relieves the nuclear inhibition. This second effect apparently relieves not only the inhibition imposed by the virus but also that resulting from cell-cell contact and serum deprivation. There are no data yet concerning relationships between the two influences of the virus on cellular DNA synthesis. It seems improbable, however, that the virus is simultaneously directing two opposed actions on a single cellular function. It is more likely that the early viral process that results in nuclear inhibition is waning at the later time, when the stimulatory effect on the nucleus is generated.

Morphological alteration and loss of contact inhibition became recognizable at about the time that nuclear DNA synthesis increased in infected cells. All of these effects could be the result of virus-controlled modification of the cell membrane. This would fit with accumulating evidence that there are contact-sensitive determinants on the surface of mammalian cells that regulate both cell movement and cell multiplication. Additional evidence suggesting that fibroma virus causes distinct changes in cell membranes comes from study of surface antigens. Several recent studies have demonstrated that pox viruses, including fibroma virus, cause antigenic changes in the surface of infected cells (15, 19; W. Tompkins and W. Rawls, *Bacteriol. Proc.*, p. 199, 1969).

In some respects, the fibroma virus-DRK cell system is similar to *in vitro* infections by SV40 virus or polyoma virus. There are a number of reports describing induction of cellular DNA synthesis by SV40 virus or polyoma virus in contact-inhibited and x-irradiated cultures (5-7, 13). The data suggest that these viruses code for a single function, possibly production of a surface antigen, which is expressed both as a change in contact inhibition and induction of cellular DNA synthesis in transformed cells. However, the response of DRK cells to fibroma virus differs significantly from cell transformation induced by other viruses or by other oncogenic agents in respect to the requirement for cell division prior to manifestation of the transformed state. Chang and Sinskey (4) found that human amnion cell cultures irradiated with 1,000 r failed to develop transformed foci after infection by SV40 virus. Other studies of the effects of chemical carcinogens, x-irradiation, and SV40 and Rous sarcoma viruses indicate

that one or more cycles of cell division are required for morphological expression of cell transformation (1-3, 17, 18), whereas morphological alteration seen in DRK cells after infection by fibroma virus occurs even though cell division is blocked by gamma radiation, or cell crowding and serum deprivation.

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