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RESPONSE OF CULTURED RABBIT CELLS TO INFECTION WITH THE SHOPE FIBROMA VIRUS

I. PROLIFERATION AND MORPHOLOGICAL ALTERATION OF THE INFECTED CELLS

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ABSTRACT

HINZE, HARRY C. (University of Wisconsin, Madison), AND DUARD L. WALKER. Response of cultured rabbit cells to infection with the Shope fibroma virus. I. Proliferation and morphological alteration of the infected cells. J. Bacteriol. 88:1185-1194. 1964.—Primary and serially cultured rabbit kidney cells were grown under conditions promoting rapid cell multiplication. When such cultures were infected with 5 to 10 plaque-forming units of Shope fibroma virus, cell multiplication was inhibited for a period of 2 to 3 days. After this stationary period, cell multiplication in the infected cultures was resumed at a rate approximating that of the uninfected controls. With the resumption of cell multiplication in the infected cultures, concurrent changes were observed in cell morphology and growth pattern. Cells showing such alterations also possessed the ability to form tumors when inoculated into the hamster cheek pouch.

Increased interest in the study of virus-induced transformation of cultured cells by animal tumor viruses has brought to light several examples of this phenomenon. Observations on the response of mouse and hamster embryo cells to infection with the S.E. polyoma virus indicate that prolonged infection with this virus gives rise to a variety of new cultural characteristics which remain stable through repeated cell division (Vogt and Dulbecco, 1960; Sacks and Medina, 1961). Similar changes occur also in cultured human and hamster cells infected with simian virus SV_{40} (Koprowski et al., 1962; Shein and Enders, 1962; Black and Rowe, 1963), and in fetal bovine conjunctiva cells infected with the bovine papilloma virus (Black et al., 1963). Such cultures are termed "transformed" cultures, and exhibit differences in morphology, rate of multiplication, and growth pattern from those of the noninfected cells. In some instances, the transformed cells are capable of tumor formation when reintroduced into the living animal host (Vogt and Dulbecco, 1962; Black and Rowe, 1963). A similar but less dramatic change characterized by an increase in proliferative capacity of rabbit skin cells without a noticeable change in morphology occurs after in vitro infection with Shope rabbit papilloma virus (Coman, 1946; DeMaeyer, 1962). Such cells also produced tumors when returned to the donor rabbit (Coman, 1946).

A somewhat analogous change is seen in the morphological conversion of cultured chick-embryo cells infected with Rous sarcoma virus (Manaker and Groupé, 1956; Temin and Rubin, 1958), and in embryonic and adult chicken cells infected with avian myeloblastosis virus (Baluda and Goetz, 1961). Unlike the transformation by polyoma and SV_{40} viruses, the conversion by these two viruses occurs rapidly, in 1 to 3 days with Rous sarcoma and in 5 to 7 days with avian myeloblastosis, and is usually associated with continuous production of large amounts of virus by the altered cells (Temin, 1962; Baluda, 1962).

A preliminary report from this laboratory (Hinze and Walker, 1963) indicated that the response of rabbit kidney cells to infection with Shope fibroma virus under carefully controlled conditions permits the continued proliferation of infected cells, together with a simultaneous alteration in cell morphology and loss of contact inhibition resembling, in some respects, that seen with the systems described above. The purpose of this report is to enlarge and extend the observations presented earlier.

MATERIALS AND METHODS

Virus. The Patuxent strain of Shope rabbit fibroma virus was obtained from the American Type Culture Collection. In this laboratory, the

virus has been maintained by intratesticular inoculation in domestic rabbits and in vitro in primary and serially cultured rabbit kidney cells. Stock virus for these experiments was prepared by inoculating serially cultured rabbit kidney cells (DRK line) in growth medium and harvesting the infected cells and fluid after 4 to 5 days of incubation at 35 C. The crude suspension was sonically treated in a Mullard ultrasonic disintegrator to release cell-bound virus, and was then stored at −70 C. The titer of virus prepared in this way ranged between 10^6 and 10^7 plaque-forming units (PFU) per milliliter.

Cell cultures. Primary cell cultures were routinely prepared from the kidneys of 2- to 3-week-old albino rabbits according to previously described methods (Padgett, Moore, and Walker, 1962). Secondary cultures prepared from 4- to 5-day primary monolayers were used in a portion of these experiments.

A serially cultured line of rabbit cells (DRK) has been developed from the kidneys of New Zealand Whites, and has been used continually in this laboratory for the past 2 years. Although the stock DRK cell line does not represent a clonal isolate, the culture consists of a fairly homogeneous population of epithelial-like cells growing in a uniform monolayer. Both the primary and serially cultured cells were propagated in a medium of 199 (Morgan, Morton, and Parker, 1950), 0.25% lactalbumin hydrolysate, and 10% heated calf serum. Penicillin and streptomycin were added in respective concentrations of 100 units and 100 μg per ml of medium. Stock DRK cells were grown in 6-oz prescription bottles (Moderne Ovals, Foster-Forbes Glass Co., Marion, Ind.), and were transferred at weekly intervals to maintain a working stock of cells.

Cell growth curves. Multiplication of control and fibroma-infected rabbit kidney cells was determined in the following way. Actively growing stock cultures were dispersed by treatment with a mixture of 0.06% trypsin and 0.1% sodium ethylenediaminetetraacetate (EDTA) in a calcium- and magnesium-free salt solution (Marcus, Cieciura, and Puck, 1956). The cells were sedimented by centrifugation, resuspended in growth medium, and seeded on cover glasses in flat-bottom shell vials (21 by 70 mm; Corning Glass Co., Corning, N.Y.). The vials were routinely seeded with a concentration of 10^5 cells in 2.0 ml of growth medium, and were incubated overnight to allow attachment to the glass. Groups of experimental cultures were then infected with fibroma virus in a concentration of 5 to 10 PFU per cell. This inoculum was sufficient to infect approximately 100% of the cells in 24 to 48 hr. Infected and uninfected control groups were incubated at 37 C in growth medium. All cultures were refed at 1- to 2-day intervals to avoid exhaustion of the medium and excessive pH change due to cell metabolism. Cell multiplication was determined by counts at daily intervals for the first 4 days and at 2- to 3-day intervals thereafter for periods up to 4 weeks. Each count was made on the pooled cells of three vials from each experimental group. Cells were prepared for counting by removal from the glass with trypsin-EDTA at 37 C. The pooled cell suspensions were chilled in an ice bath and immediately counted in a hemocytometer.

Virus assay. Plaque assays were carried out on monolayers of DRK cells grown in 1-oz prescription bottles. Bottles were inoculated with 0.5-ml amounts of appropriately diluted virus and incubated at 37 C for 4 hr to permit adsorption of the virus. After the adsorption period, the cells were covered with an overlay composed of Hanks' balanced salt solution (BSS, without phenol red) containing 0.5% lactalbumin hydrolysate, 0.1% bovine albumin, 0.1% yeast extract, 6% horse serum, and 1.5% methylcellulose (Dow Chemical Co., Midland, Mich.); 5 ml of overlay were added to each bottle, and the cultures were incubated at 35 C for 7 to 10 days to obtain plaques 1 to 2 mm in diameter. The semisolid overlay was then withdrawn, and the cell layer was covered with a fixative composed of ethyl alcohol (6 parts), glacial acetic acid (2 parts), and 40% formaldehyde (1 part). After fixation for 2 to 3 min or longer, the cell sheet was washed quickly in a forceful stream of tap water and allowed to drain dry. Plaques were easily counted on the unstained cell sheet when viewed by oblique light over a dark background.

The sensitivity of this method for assay of fibroma virus was found comparable to that obtained by titration on monolayers of primary rabbit kidney cells under agar (Padgett et al., 1962) and by intradermal inoculation into rabbits.

RESULTS

Proliferation of infected DRK cells. Multiplication of rabbit kidney cells after infection with the Patuxent strain of fibroma virus was shown by

comparison of the growth curves of infected and noninfected cells in the following experiment. Replicate cultures of DRK cells were prepared in shell vials. After incubation overnight at 37 C, one group of cultures, containing approximately 10^5 cells per vial, was inoculated with 10^6 plaque-forming units of virus contained in 1.0 ml of growth medium. A similar group of cultures was given an equal amount of growth medium and held as uninoculated controls. Both groups of cultures were incubated at 37 C and refed with 2.0 to 3.0 ml of growth medium at 2-day intervals for the first 8 days and daily thereafter for the following 14 days. At the intervals indicated in Fig. 1, cell counts were done on both infected and control groups. At the same intervals, the infected cultures were examined for virus content by plaque assay, for viral antigen by fluorescent-antibody staining, and for morphological changes by staining with eosin and hematoxylin. Multiplication of the control cultures was preceded by a lag period of 24 hr. Maximal mitotic activity was apparent for the next 3 days, during which period the generation time of the uninfected cells was 30 to 36 hr. Increased crowding in the culture after this time reduced cell proliferation, and resulted in a stable population for the remainder of the experimental period.

Introduction of fibroma virus into the cultures 24 hr after seeding extended the lag period 72 hr beyond that of the control cells. An increase in the number of mitotic figures seen in histological stains made 72 hr after addition of virus was reflected in an increased cell number beginning 24 hr later and continuing for the next 7 days. Minimal generation time for the infected cells during this period was 48 hr. After reaching a maximal population of 6×10^5 cells per vial on day 12, growth of the infected cultures remained stationary until the experiment was terminated 11 days later. Although Fig. 1 represents a single growth curve experiment, comparable results were obtained in numerous similar studies with this strain of fibroma virus in DRK cells.

Virus multiplication. The development of cell-associated virus in DRK cells is shown in Fig. 2. Starting at 4 hr after infection and thereafter at the indicated intervals, samples of infected cells were washed three times with BSS and disrupted by ultrasonic vibration; the virus content was determined by plaque assay. Between 4 hr and 2 days after infection, the titer of cell-associated virus rose sharply to a peak of 2×10^6 PFU;

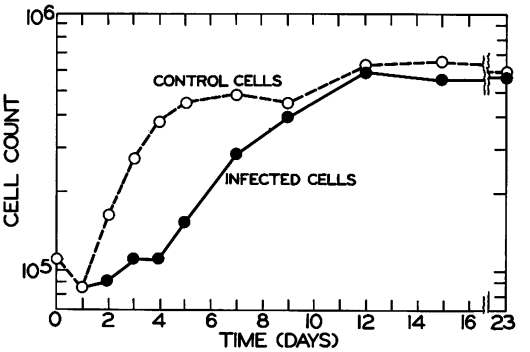

FIG. 1. *Growth of control and fibroma-infected DRK cells. Cells were seeded on day zero and infected 24 hr later (day 1). Cell count represents average total cells per culture vial.*

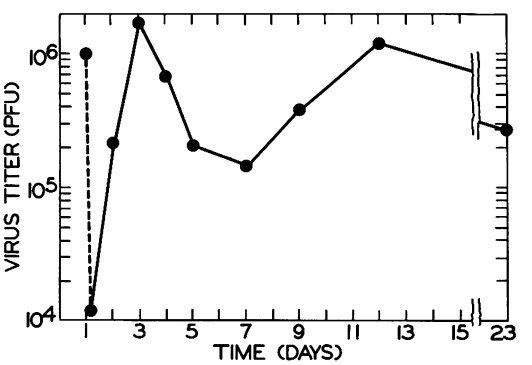

FIG. 2. *Development of cell-associated virus in fibroma-infected DRK cells (from Fig. 1). Virus titer represents total cell-bound virus per culture vial. Initial inoculum contained approximately 10 plaque-forming units per cell.*

the cell count during this period remained stationary at 10^5 cells per vial. An estimate of the fraction of cells infected at this time was made by examination of duplicate cultures stained with fluorescent antibody, and showed that essentially all cells contained large amounts of viral antigen by 48 hr. Beginning on the third day of infection, the concentration of virus dropped rapidly and remained at a relatively low level throughout the period of most active cell proliferation, rising again as the cell population reached a plateau and declining slowly thereafter. The variation in cell-bound virus was paralleled by similar changes in the amount of viral antigen visualized by fluorescent-antibody staining.

Morphology of infected DRK cells. Changes in cell morphology which occurred in the infected

cultures are illustrated in Fig. 3 through 7. Numerous cytoplasmic inclusions were evident in the infected cells 24 to 48 hr after addition of the virus (Fig. 3). Beginning at about 3 days postinfection, and coincident with the onset of mitosis, cells of the virus-containing cultures became larger and assumed a more distinct spindle shape than the control cells (Fig. 4).

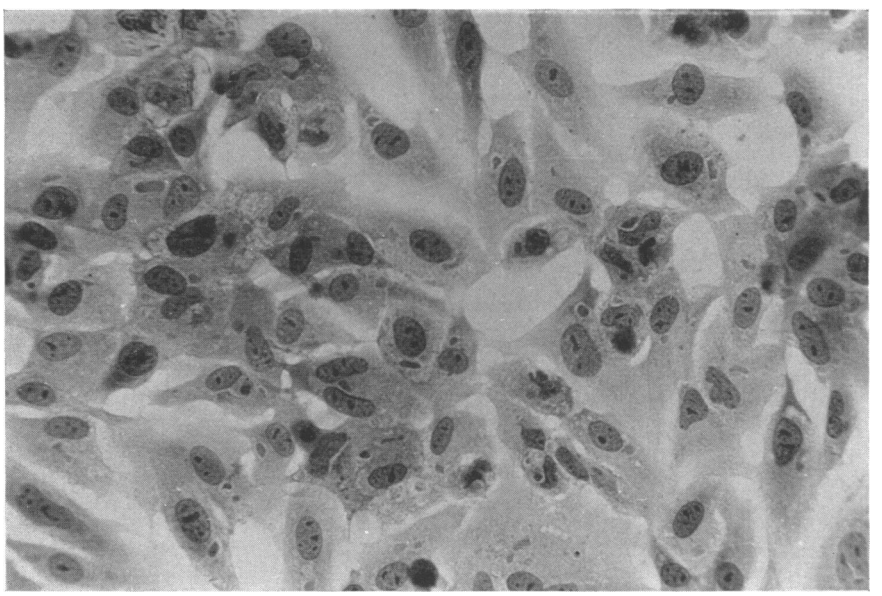

FIG. 3. *DRK cells 48 hr after addition of fibroma virus. Typical cytoplasmic inclusions appear at this time in virtually all cells of the infected culture. No other changes in cell morphology are apparent at this time. Eosin and hematoxylin stain.* ×*160.*

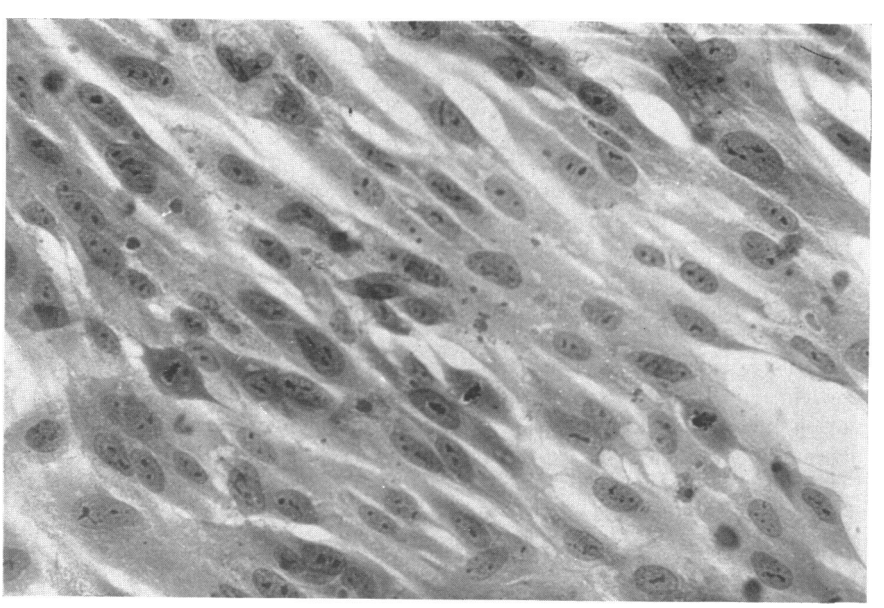

FIG. 4. *Infected DRK cells assume a distinct fibroblast-like appearance 3 days after addition of virus. Increased cell division is evident from the numerous mitotic figures. Eosin and hematoxylin stain.* ×*125.*

As the infected cultures continued to multiply, the growth pattern of these cells developed obvious differences from that of the controls. Loss of contact inhibition at this time was indicated by the random arrangement of cells to form a crisscross network, as illustrated in Fig. 5. Upon further incubation, these cultures developed dense, interwoven cords of cells several layers thick (Fig. 6). In contrast, control cultures maintained under the same conditions remained in a

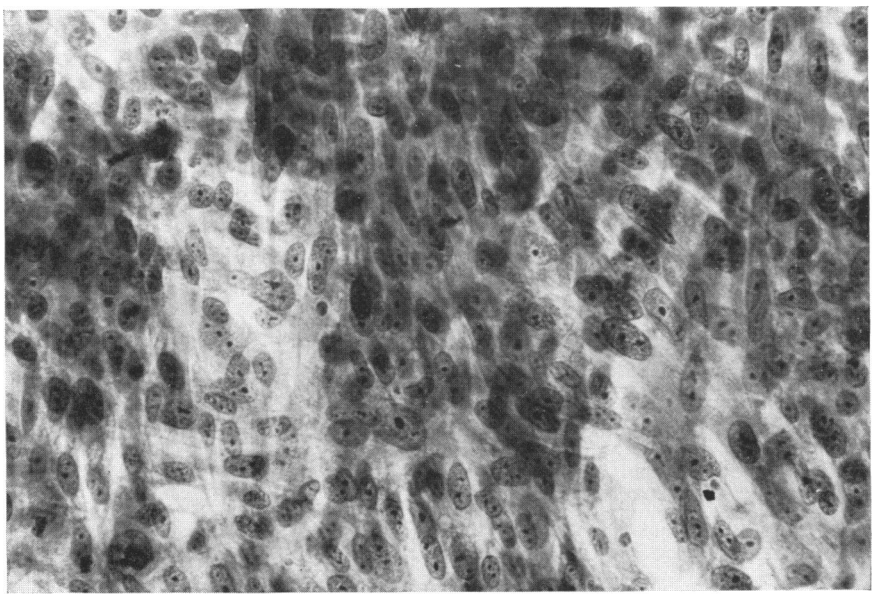

FIG. 5. *Loss of contact inhibition is obvious in this culture of DRK cells infected 6 days previously. Eosin and hematoxylin stain. ×125.*

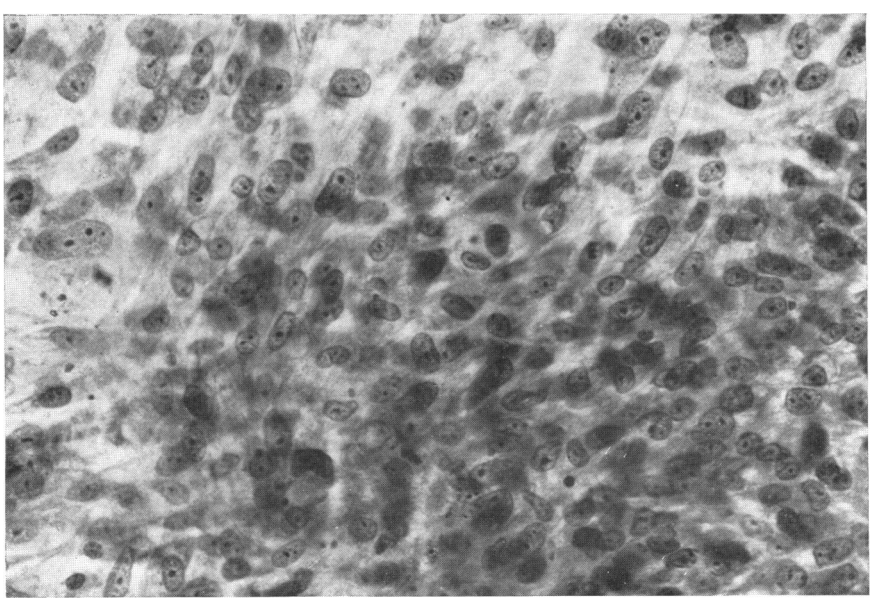

FIG. 6. *DRK culture 14 days after infection shows typical multilayering of cells. Cytoplasmic inclusions are less frequently seen than in earlier stages of infection. Eosin and hematoxylin stain. ×125.*

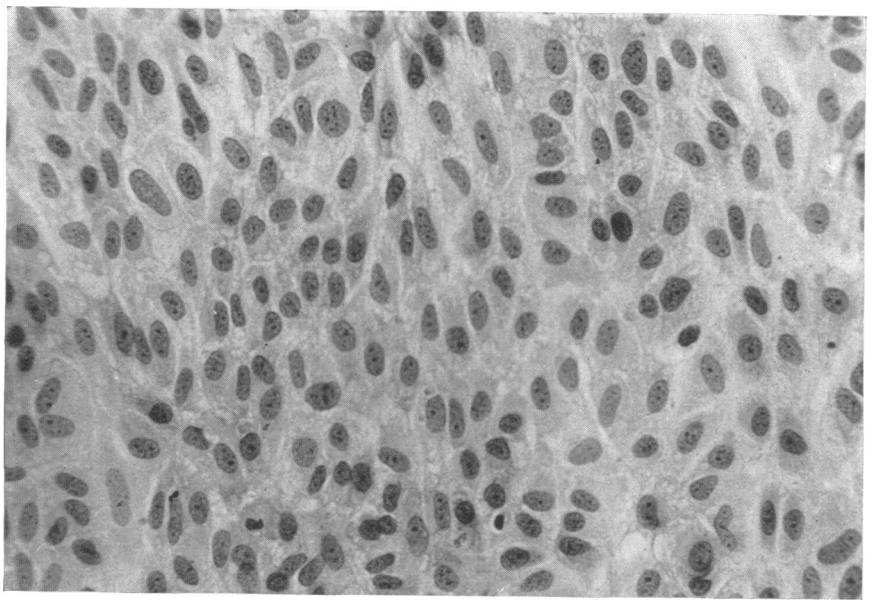

FIG. 7. *Control culture of DRL cells. Uninfected cells show uniform monolayer growth throughout experimental period. Eosin and hematoxylin stain.* ×*125.*

uniform monolayer throughout the experimental period (Fig. 7).

Further indication of the change in morphology of the infected cells was obtained by comparing the average measurements of the long and short axis of individual infected and control cells. Measurement of several hundred uninfected DRK cells in a confluent monolayer 11 days after seeding gave an average length of 35 μ and an average width of 17 μ (length-width ratio of 2:1); infected DRK cells of a heavily populated culture of the same age averaged 70 μ long and 10 to 11 μ wide (length-width ratio of 7:1).

Maximal cell density. Results of the previous experiment have shown that the maximal cell density of control cultures remained slightly above that of infected cultures, despite the fact that growth of the control cells was restricted to a monolayer while the infected cells multiplied to a depth of three to four cell layers in most areas of the culture. This difference in maximal cell density between control and infected cells has been observed repeatedly and often has been greater than indicated in Fig. 1. Two factors contribute to this apparent discrepancy. (i) Control cells in a crowded monolayer appear considerably smaller than infected cells of a similar age. Each control cell, therefore, requires relatively less space than its infected counterpart. (ii) Control cells grow first on the surface of the cover glass contained in the culture vial and then migrate off the cover glass to grow up the vertical wall of the vial. Such cells often grow to a height of 3 to 4 mm up the wall of the vial. Infected cells, on the other hand, grow only on the surface of the cover glass. These cells show no tendency to spread to the inside wall of the vial and are, therefore, more limited in available surface area. To determine the maximal cell density reached by the control and infected cultures when both are restricted to the same surface area, the following experiment was performed. The inside surface of a number of shell vials was coated to a depth of 2 to 3 cm with a thin film of silicone lubricant (Dow Corning Corp., Midland, Mich.) dissolved in ether. The vials were thoroughly dried, and cover glasses were inserted into the bottom of each. The coated vials were then seeded with approximately 10^5 DRK cells and infected as in the previous experiment. Cell counts were performed at the times indicated in Fig. 8. Microscopic examination of the vials showed that both control and infected cells grew as usual on the surface of the cover glass, but were completely prevented from growing on the wall of the vial. The results (Fig. 8) showed a definite limit in the number of control cells (5×10^5) which could grow on the surface area provided by the cover

slip. The infected cultures, however, grew to a maximal concentration of 6.5 × 10^5 cells under identical conditions. The initial drop in cell number after infection was possibly due to some slight toxic effect of the coating, although a similar drop has occasionally been observed as well with uncoated vials.

Effect of fibroma infection on primary cells. Figure 9 illustrates a typical growth curve obtained after infection of primary rabbit kidney cells. Shell vials seeded with 10^5 primary kidney cells were inoculated 24 hr later with 6 PFU of fibroma virus per cell in the same manner as in the previous experiments. Control cultures were maintained in growth medium without virus. Multiplication of the uninoculated primary cells proceeded in a manner similar to that of the DRK controls (Fig. 1). After a lag phase of 24 hr, the cells multiplied at a logarithmic rate to a plateau of approximately 6 × 10^5 cells per vial. Minimal division time for the control cells during this period was 36 hr. Addition of fibroma virus to the primary cell cultures extended the lag period 24 hr beyond that of the controls. Cell multiplication then proceeded somewhat more rapidly than in the uninfected cells with a minimal generation time of 32 hr. The maximal cell density was the same for both infected and control groups at approximately 8 × 10^5 cells per vial. Although the primary cultures were composed of a variety of cell types, the infected group demonstrated changes in morphology and growth pattern consistent with those seen in the infected DRK cultures (Fig. 10 and 11).

Tumor formation in hamsters. A preliminary study of the ability of fibroma-infected primary and serially cultured rabbit cells to produce tumors when reinoculated into the homologous host gave inconclusive results owing to the continued presence of infectious virus. For this reason, inoculation into the hamster cheek pouch was used to demonstrate this property.

DRK cells grown in 6-oz prescription bottles were infected with fibroma virus at a concentration of 5 PFU per cell and incubated 8 days at 37 C with frequent medium changes. The cells were then removed from the glass by trypsin-EDTA treatment, washed, and resuspended at the desired concentration in BSS. Groups of eight 25-day-old Syrian hamsters were inoculated, under ether anesthesia, in the cheek pouch with suspensions of 10^5 or 2 × 10^6 cells contained

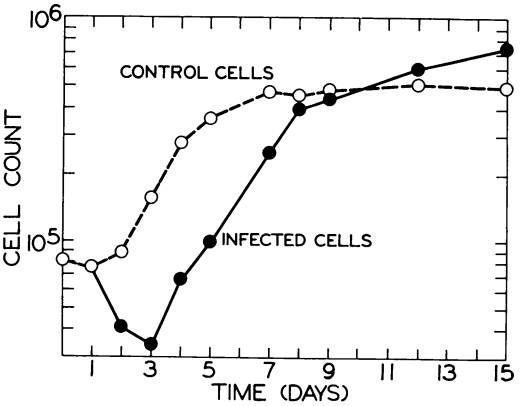

FIG. 8. *Maximal cell density obtained by control and fibroma-infected DRK cells growing on a restricted surface area. Control cells are sharply limited in growth when monolayer covers the entire available area. Infected cells with the same available area continue growing in a multilayer.*

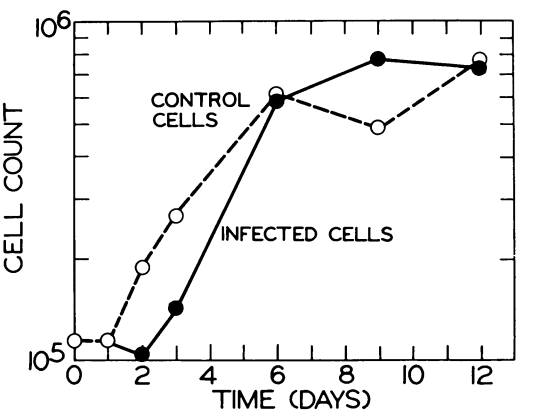

FIG. 9. *Growth of control and fibroma-infected primary rabbit kidney cells. Multiplication of the infected cells approximates that of controls after an additional 24-hr lag period.*

in a total volume of 0.2 ml. Control groups were inoculated with equal numbers of uninfected cells, and one group of four animals was given 10^7 PFU of stock fibroma virus. Only those animals receiving 2 × 10^6 infected cells showed evidence of tumor formation. In this group, seven of the eight animals developed a single small white nodule 4 to 5 days after inoculation. The tumors developed a maximal diameter of 5 to 7 mm in 7 to 10 days and regressed completely in 4 to 5 weeks. Tumors removed 2 weeks after inoculation appeared grossly as firm, white, well-circumscribed masses having a fibrous consistency

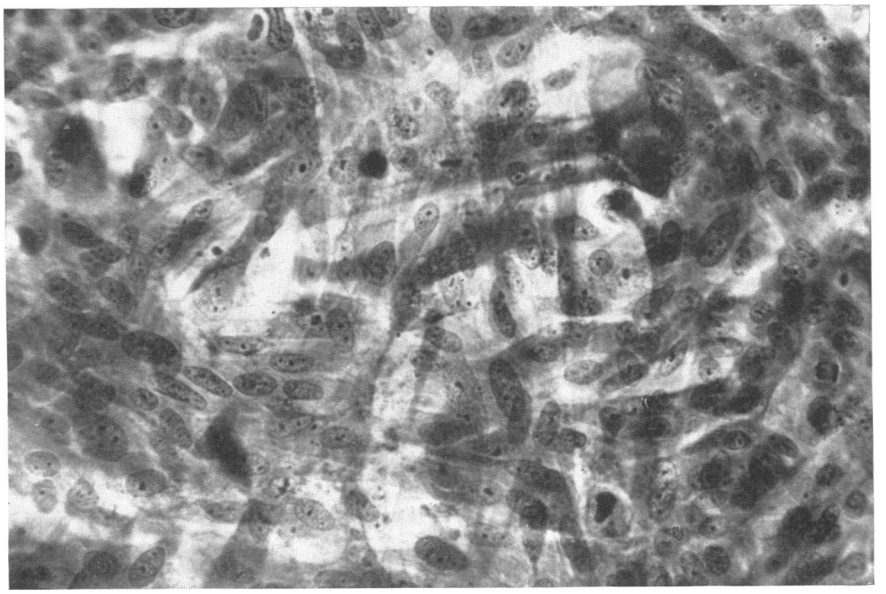

FIG. 10. *Primary rabbit kidney culture 6 days after infection with fibroma virus. Cells show changes in morphology and growth pattern similar to those seen with infected DRK cells. Eosin and hematoxylin stain. ×125.*

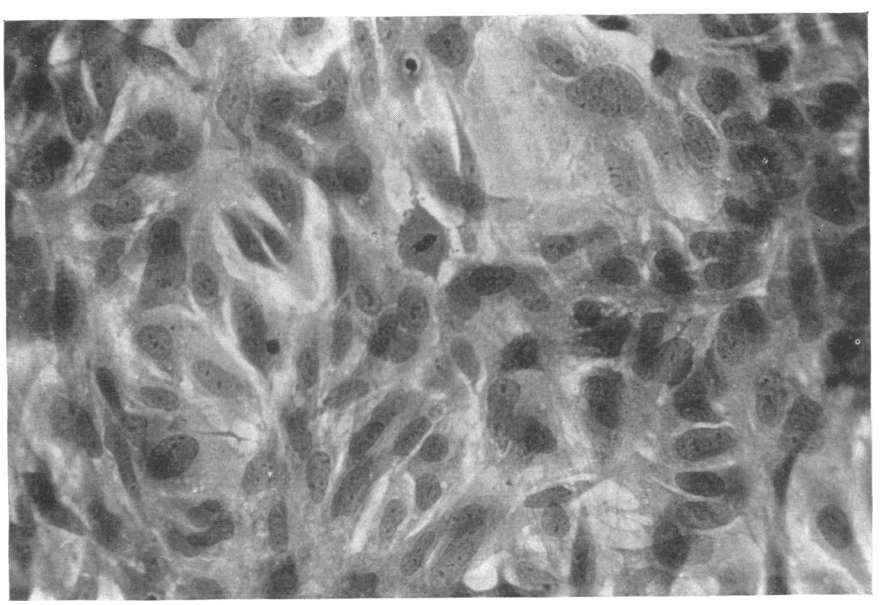

FIG. 11. *Control culture of primary rabbit kidney cells. Eosin and hematoxylin stain. ×160.*

on cutting. No evidence of necrosis or abscess formation was evident at any time. Histological studies are in progress and will be reported later. Animals receiving control cells, virus, or 10^5 infected cells showed no visible reaction to the inoculation.

DISCUSSION

Previous studies by other workers on the response of cultured rabbit cells to infection with Shope rabbit fibroma virus have resulted in a variety of contrasting observations. In studies by Constantin, Febvre, and Harel (1956), it was

observed that roller tube cultures of rabbit spleen tissue failed to show significant cell destruction while infected with fibroma virus. In more recent studies, Febvre (1962) reported an early "luxuriant" growth of fibroma-infected rabbit testis cells followed by a cytopathogenic effect in 6 to 8 days. Kilham (1956) similarly described infection in cultured cottontail and in domestic rabbit testis cells (Kilham, 1957) without resulting cytopathogenic effect; in later studies the same worker (Kilham, 1958, 1959) demonstrated a partial cytopathic effect (CPE) resulting from fibroma infection in cultures of domestic rabbit kidney cells. A similar partial CPE due to fibroma virus was seen in rabbit kidney cells maintained under conditions of reduced nutrition in this laboratory (Padgett et al., 1962). Other workers (Chaproniere and Andrewes, 1957; Verna and Eylar, 1962) observed a marked CPE of rabbit kidney cells as a result of fibroma virus infection. Such disparity most probably reflects differences in a number of variable factors, e.g., type of host cell and virus strain used, initial multiplicity of infection, and environmental conditions under which the infected cells were maintained. In none of the previous studies was an attempt made to maintain the infected cultures under conditions optimal for cell proliferation.

In the present report, examination of the response of primary and serially cultured rabbit kidney cells to infection with the Patuxent strain of fibroma virus has shown that, when conditions of nutrition and environment are maintained at an optimum, the infected cells will multiply extensively and display a significant change in morphology and growth pattern. The change in the infected cells occurs consistently within 3 to 4 days after infection, usually without noticeable cell destruction, and together with active production of infectious virus. In these respects, the effect of fibroma infection on rabbit cells described here resembles more closely the morphological conversion of cultured chick-embryo cells by Rous sarcoma virus than other types of virus-mediated cell transformation so far reported in the literature. Such a comparison is interesting when one considers that it occurs between viruses of different size, structure, and nucleic acid composition. As with the Rous sarcoma system, the alteration in morphology and loss of contact inhibition are most logically explained as a result of the controlling influence of the virion at the cell surface or at some position inside the cell (Vogt and Rubin, 1962; Temin, 1962). This is in sharp contrast to the cell transformation by other deoxyribonucleic acid tumor viruses, which is characterized by the disappearance of detectable virus or viral components in the altered cells (Dulbecco, 1962).

It is also possible that the observed phenomenon represents the emergence of a minor morphological component through the selective pressure of massive viral infection. Studies with cloned cell lines are now in progress to gain a more complete understanding of the importance of such selection.

The neoplastic growth of virus-transformed cells upon introduction into a suitable animal host has been observed with some degree of regularity (Vogt and Dulbecco, 1962; Black and Rowe, 1963; Coman, 1946; Temin, 1962). In the case of polyoma-transformed hamster cells, acquisition of this characteristic of malignancy has been described as a secondary variation following an early alteration in morphology and growth pattern (Vogt and Dulbecco, 1962). The ability of fibroma-infected rabbit kidney cells to multiply and form tumors when inoculated into the hamster cheek pouch indicates the acquisition of an additional character by the infected cells; however, the relationship of this property to the possible malignant nature of these cells will require further study.

ACKNOWLEDGMENTS

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