Induction of Neoplasia *in Vitro* with a Virus

Experiments with Rabbit Skin Grown in Tissue Culture and Treated with Shope Papilloma Virus*

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Since neoplasia has been induced in tissue culture by means of a chemical carcinogen (5), the question arises whether similar neoplastic transformation of cells *in vitro* can be brought about by other types of carcinogens, such as viruses.

This question was answered by successfully growing epidermis of the domestic rabbit in tissue culture and then inoculating it with the Shope papilloma virus. The experiments to be reported show that under these conditions, neoplastic transformation of epithelial cells occurs in *vitro*.

**MATERIAL AND METHODS**

The method of tissue culture consisted in embedding skin fragments in streaks of plasma on the side of test tubes. This method as previously described (3) was slightly modified because of difficulties inherent in cultivating skin epithelium.

The back of a domestic rabbit was shaved with a razor as closely as possible. The exposed skin was then scrubbed with warm water and soap daily for 4 days. On the fourth day the skin was washed with 95% alcohol, after which an elliptical piece about 3 cm. long was removed surgically and placed in physiological saline solution. No attempt was made to separate the epidermis from the underlying connective tissue. The skin was cut into fragments 2-3 mm. in diameter and these were embedded in a streak of chicken plasma in an ordinary test tube. The plasma was clotted with embryo extract, and a fluid medium consisting of human umbilical cord serum diluted with saline solution was added. In the saline solution was dissolved aspartic acid (0.3%), penicillin (1500 units per cc.), and sulfanilamide (2.0%). The final fluid medium, for each tube, consisted of 10 drops of serum, 10 drops of salt solution containing the aspartic acid and penicillin, and 10 drops of salt solution containing the sulfanilamide. The tubes were then placed in a rotator (4) housed in an incubator at 37° C.

To such cultures papilloma virus was added. Papillomas 1 from cotton-tail rabbits were ground in a mortar with sand to form a smooth paste. The paste was extracted with 10 volumes of physiological salt solution for 12 hrs. This suspension was then centrifuged and the supernatant fluid passed through a Berkefeld N filter. The resulting clear, sterile, cell-free filtrate was tested for potency in the skin of domestic rabbits before using in the tissue cultures. Usually 6 drops of active virus preparation were added to each culture tube. The fluid medium was changed every third or fourth day.

**RESULTS**

Cultures without papilloma virus, after 3 to 5 days, showed small clusters of squamous epithelial cells at the edges of the skin fragments. These clusters slowly enlarged to form small sheets of cells (Fig. 1). Growth was never rapid or luxuriant and nearly ceased after a week or 10 days, with slow deterioration of the cultures thereafter.

In contrast, when papilloma virus was added to such cultures a burst of epithelial proliferation followed within one or two days and vigorous growth persisted for 2 to 3 weeks (Fig. 2). Much larger sheets of epithelial cells were formed which eventually broke up into smaller clusters. Cytological studies upon untreated and upon virus-treated cultures were made in hanging drops to allow the use of higher power lenses. No significant changes were noted in the cells from virus-treated cultures.

The increased growth activity of the cultures following introduction of the virus suggested the probability that neoplasia was being induced *in vitro*. To test this probability further, explants were removed from the tubes, washed in saline and implanted in the livers of domestic rabbits as follows:

Virus-treated cultures of skin were implanted in the liver of the same domestic rabbit from which the skin

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1 The papillomas used as a source of virus were obtained through the kindness of Drs. Richard Shope and Peyton Rous, of the Rockefeller Institute for Medical Research, and Dr. Joseph Beard, of Duke University.
Fig. 1.—A small sheet of epithelial cells in a tissue culture of rabbit skin. This culture was not treated with papilloma virus. The dark mass on the left is the original explant; the group of paler cells near the center of the figure is the outgrowth. This culture was five days old. The photomicrograph was taken through the wall of the culture tube and the cells were not fixed or stained. Mag. X 225.

Fig. 2.—A large sheet of epithelial cells in a tissue culture of rabbit skin to which was added papilloma virus. The picture was taken on the fourth day of culture. Mag. X 110.

Fig. 3.—Fibrosis surrounding a degenerating clump of squamous epithelium in the liver. The skin, in this case, had been removed from the same rabbit into which it was later implanted. Meanwhile it had been grown in tissue culture without the addition of papilloma virus. Mag. X 72.
had been obtained originally, as well as in another rabbit. Six pairs of rabbits were so treated. Another group of 6 pairs of rabbits served as controls. Cultures of skin not treated with virus were planted in the rabbit from which the skin had been removed, and also in another rabbit. Also, virus alone was injected into the livers of 6 domestic rabbits.

The implants were made by exposing the liver of the anesthetized rabbit through an abdominal incision. Small slits were made in the liver with the point of a scalpel, and the tissue culture explants were tucked into these slits with a pair of forceps. The incision was then closed by suture, and the rabbits were kept for from 3 to 8 weeks, after which they were sacrificed and the implantation sites examined.

In rabbits in which virus alone had been injected into the liver no lesions of any kind were found, nor were any expected, since it is well established (7) that the papilloma virus is specific for the skin epithelium of the rabbit.

Rabbits bearing implants of skin which originally came from other rabbits, whether the culture of the skin had been treated with virus or not, showed no tumors. The implanted fragments in some cases had persisted as small white flecks surrounded with scar tissue, and microscopic sections (Fig. 3) revealed fibrosis and an occasional nest of degenerating epithelial cells.

In contrast, rabbits bearing implants of their own skin which had been treated with papilloma virus showed regularly, opaque, white, solid nodules of tumor tissue 2.5 to 3 cm. in diameter, at the sites of implantation. These nodules were many times the size of the original explants (Fig. 4). Microscopic sections of these nodules revealed masses of proliferating squamous epithelium containing many mitotic figures (Fig. 5). As shown in the photomicrographs (Figs. 6-7), the proliferating epithelium formed a papillomatous pattern even when growing in the liver. These growths were similar to those obtained when papillomas from the skin are transplanted into the liver of the same rabbit (7).

**DISCUSSION**

The foregoing experiments indicate that the rabbit papilloma virus is capable of inducing neoplasia in vitro. The only other experiments in which neoplasms were produced by a virus in vitro are those of Carrel (1) and Carrel and Ebeling (2), using the Rous sarcoma. They concluded that the virus was present in macrophages, which they thought to be the essential cells constituting the Rous sarcoma.

When, in the present experiments, tissue cultures of rabbit skin were treated with papilloma virus and later implanted in rabbit livers, tumors resulted only when the skin was implanted in the rabbit from which it had been removed originally. This indicates that the skin had not lost its individual specificity during tissue culture and is also in agreement with the observation that the tissue cells of the rabbit papilloma cannot be successfully transplanted from one rabbit to another, whereas they can be transplanted into different situations in the rabbit in which they are growing (8).

It would be of interest to determine whether or not malignant growth can be induced by the papilloma virus in vitro. Malignant change of the papilloma occurs in the domestic rabbit in a considerable proportion of cases when it is allowed to grow for a sufficient period (9). To determine the occurrence of malignancy in vitro one might observe the behavior of long-term tissue cultures subsequently implanted in rabbit livers, using invasive growth and metastases as indication of malignancy. Also one might use heterologous intra-ocular transplants (6) as a test for malignancy. Heterologous transplantations are successful only with malignant tumors, so that the benign papilloma should fail to grow in the eye of an alien species, whereas the squamous cell carcinoma should grow.

Other problems might well be explored by using the papilloma virus in vitro. For example, since the domestic rabbit bearing papillomas is highly resistant to further treatment with the virus (10), would skin removed from such a rabbit and grown in tissue culture remain resistant to the virus? As it is difficult to recover the virus from the papillomas of domestic rabbits (10), would it be possible to recover it from tissue cultures of domestic rabbit skin?

The use of papilloma virus and rabbit skin in vitro should serve as an excellent method for investigations of virus-cell relationships.

**DESCRIPTION OF FIGURES 4 AND 5**

**Fig. 4.**—Nodules of tumor in the liver of a rabbit. These nodules developed from implanted tissue culture fragments of rabbit skin which had been treated with papilloma virus. The implants were made into the liver of the same rabbit from which the skin had been taken. Three weeks after implantation, the fragments have grown to many times the size of the original implants.

**Fig. 5.**—A photomicrograph of a section from one of the tumor nodules illustrated in Fig. 4, showing masses of squamous epithelium which forms the bulk of the growth. Two mitotic figures are apparent. H. & E. stain. Mag. X 280.
Figs. 6 and 7.—These two photomicrographs show the papillomatous pattern characteristic of such tumor nodules as illustrated in Fig. 4. Masses of keratinized epithelium form the central parts of these tumors. The more actively proliferating cells are at the periphery. Fig. 6, Mag. × 17. H. & E. stain. Fig. 7, Mag. × 80.
SUMMARY AND CONCLUSIONS

1. Experiments were designed to determine whether neoplasia can be induced in tissue cultures of rabbit skin by papilloma virus.

2. The criteria adopted for induction of neoplasia were:
   a. Increased growth activity of epithelial cells in the cultures after introducing papilloma virus.
   b. Formation of relatively large tumors in the liver of the rabbit following implantation of such tissue cultures.

3. Both criteria were met by the experiments and it is therefore concluded that papilloma virus is capable of inducing neoplasia in vitro, namely in tissue cultures of rabbit skin.

REFERENCES

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