Significance of Continued Virus Production in Tissue Cultures Rendered Neoplastic by Polyoma Virus

Renato Dulbecco, and Marguerite Vogt

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COROLLARY. *Let the sequence of homeomorphisms $h_n(X) = Y$ converge uniformly to a mapping $h(X) = Y$. In order that h be a homeomorphism it is necessary and sufficient that the sequence (h_n) be uniformly approximately open.*

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[1] Youngs, J. W. T., "Homeomorphic approximations to monotone mappings," *Duke Math. Jour.*, **15**, 82–94 (1948).

[2] Whyburn, G. T., "Sequence approximations to interior mappings," *Am. Soc. Polon. Math.*, **21**, 147–152 (1948).

[3] Polak, A. I., "On mechanisms of uniform approximations in the domain of continuous mappings of compacta" (Russian), *Uspekhi Mat. Nauk (NS)*, **11**, No. 4 (70), 149–154 (1956).

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*SIGNIFICANCE OF CONTINUED VIRUS PRODUCTION IN TISSUE CULTURES RENDERED NEOPLASTIC BY POLYOMA VIRUS**

BY RENATO DULBECCO AND MARGUERITE VOGT

CALIFORNIA INSTITUTE OF TECHNOLOGY

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The infection with polyoma (PY) virus of monolayer cultures of either mouse or hamster embryo cells gives rise to a neoplastic transformation of the cultures.[1] Previous results showed that the transformed hamster cultures did not release any virus, whereas the transformed mouse cultures continued to release PY virus indefinitely; in the latter case, the virus released was invariably a mutant of small-plaque (*sp*) type.[2] We have since observed that in some cases transformed hamster cultures also continue to release virus indefinitely and that the virus released is also of the *sp* type.

These observations raise a number of questions: Why do transformed cultures release virus indefinitely in some cases and not in others? Why is the released virus of these cultures, once transformed, invariably the *sp* mutant? How is the release of virus related to the transformation?

To try to answer these questions, we have studied in detail the evolution of the infected cultures, the properties of the transformed cells, and the quantitative aspects of virus release from transformed mouse embryo cultures.

Results.—Growth characteristics of hamster and mouse embryo cultures transformed by PY virus: One of the main *in vitro* characteristics of transformed mouse or hamster embryo cultures is an increased "over-all growth rate," as compared to that of noninfected cultures. A measure for the "over-all growth rate," i.e. the average rate at which a culture grows when maintained by serial transfer, was obtained as follows. All the cultures were grown as monolayers in contact with the bottom of petri dishes and were transferred without delay as soon as their cells had grown into a confluent sheet. At each transfer, a known fraction of the trypsin-dispersed culture was used as the inoculum. Thus, the increment in cellular mass at every transfer was known, as well as the time required to achieve it. Plotting the logarithm of the product of all increments at successive transfers versus

the time at which the transfers were carried out supplies the "over-all growth curve" of the culture. The characteristics of the curve depend on many factors, such as the dependence of the multiplication rate of the cells on the degree of crowding of the culture, the proportion of cells surviving the transfer, the length of the lag period after transfer, etc. Cell killing by virus is not an important factor except in mouse embryo cultures during the first month following infection when cell degeneration is extensive.

Over-all growth curves for a number of cultures of hamster embryo cells infected independently with PY virus are given in Figure 1. All curves tend to a final

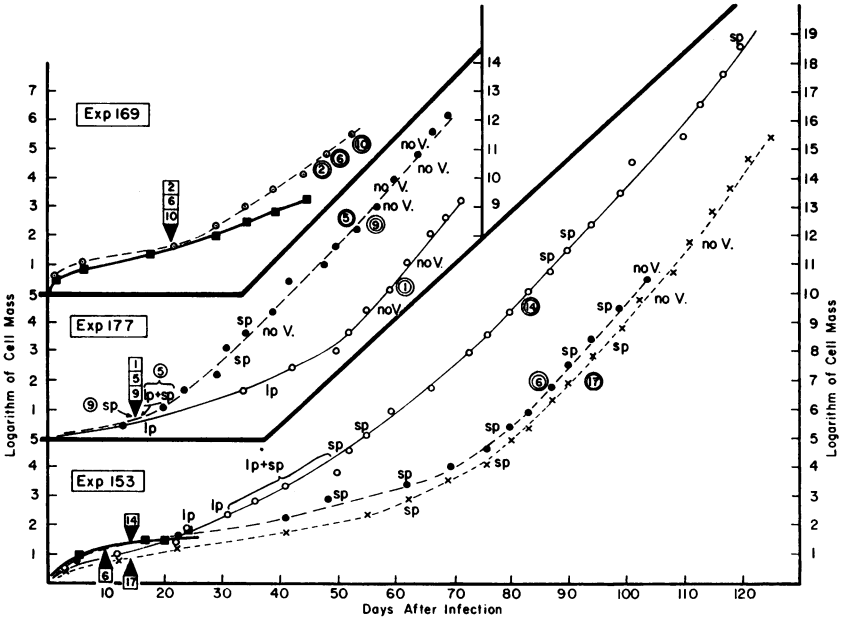

FIG. 1.—Over-all growth curve of hamster embryo cultures infected with PY virus. The heavy lines give the cell mass of uninfected cultures, the unit being the mass of a confluent sheet of cells; the thin lines give the mass of infected cultures, in the same units. The numbers in the double circles are those of the respective cultures. A large black triangle indicates the time of appearance of whorls and excessive acidification in the culture designated by the number associated with it. The designations *lp*, *sp* indicate the type of virus produced by the cultures at the corresponding time; *no V* indicates that no infectious virus was produced.

Cultures 153-14, 177-1, and 169-2 were infected with virus of *lp* type; cultures 153-6, 153-17, 177-9, and 169-10, with virus of *sp*-type; cultures 177-5 and 169-6, with an equal mixture of the two types.

increased growth rate which is approximately the same for all cultures. The increase in the growth rate occurs after the first changes observable in the culture, which consist in whorl formation and increased acidification.[1] Thus, three stages can be identified in infected hamster embryo cultures: pre-whorl, whorl, and enhanced growth rate. It is likely that the transition between the second and third stage occurs when the cells constituting the whorls—which must have an over-all growth rate greater than that of normal cells and which have been shown to be neoplastic[1]—take over the whole culture. The variability in the time required for this transition is due mostly to the technical difficulty of dispersing the whorls by trypsin.

Over-all growth curves of mouse embryo cultures given in Figure 2 also show at the final stage an increase in growth rate, probably of similar significance.

Time and mode of appearance of sp virus in cultures infected by large-plaque (lp) virus: As shown in Figure 1, most hamster embryo cultures cease releasing virus when they reach the stage of enhanced growth rate; cultures infected with *sp* virus tend to release virus for a somewhat longer time than cultures infected with *lp* virus. An exception is constituted by culture 153–14; this culture was infected with *lp* virus and continued to release *lp*-type virus during the first and second stage but changed to release of *sp*-type virus in the third stage.

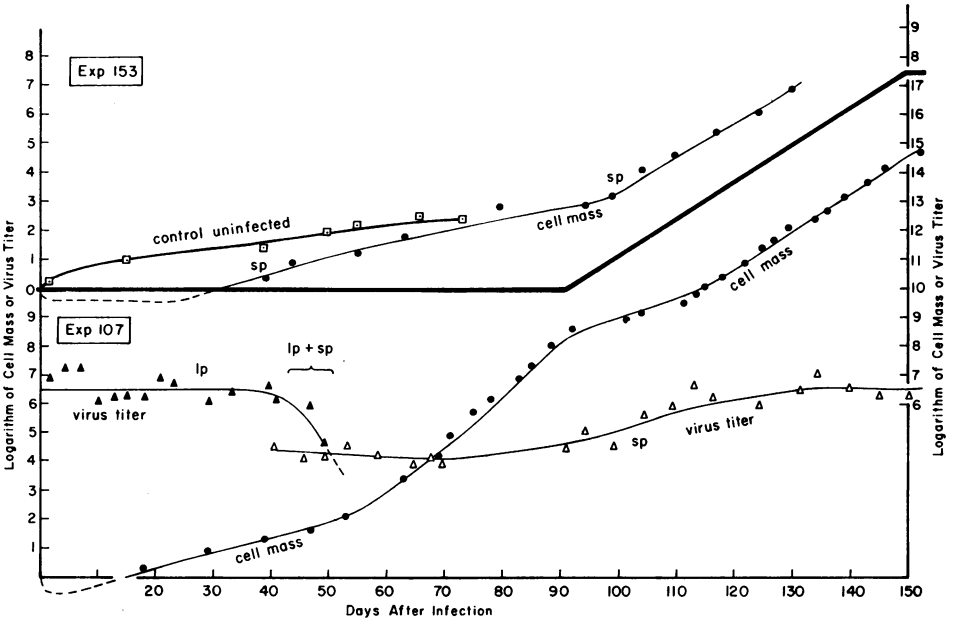

FIG. 2. Over-all growth curves of mouse embryo cultures infected with PY virus. The symbols are the same as those of figure 1. In addition, in the lower curve, ▲ = titer of *lp* virus present in the culture; Δ = titer of *sp* virus. Titers are given as PFU per culture.

The culture of experiment 107 had been infected with virus of *lp*-type; that of experiment 153 with virus of *sp*-type. The temporary decrease in growth rate of culture 107 around the 100th day was due to accidental causes.

Figure 2 shows that the infected mouse embryo cultures released virus all the time. Culture 107, which had been infected with *lp* virus, released *lp* virus until its over-all growth rate increased, and changed then, like the hamster embryo culture 153–14, to release of *sp* virus. This change in the type of virus released appears to be the usual one in mouse embryo cultures infected with *lp* virus.

These observations concerning the continued release of *sp* virus were made on the mass cultures infected by PY virus. In an attempt to evaluate its significance, virus release was studied at the level of the individual cells in the transformed, virus-releasing mouse embryo culture 107. Two questions were asked: Do all the cells of the cultures have the property of virus release? Is this property transmitted hereditarily? The two following groups of experiments answer these questions.

The distribution of the virus and of the virus yields in the cells of the virus-releasing mouse embryo line 146: To answer the first question, two experiments were carried out by using two cultures of line 146 (a derivative of line 107) showing persistent release of *sp* virus; one was an untreated culture, the other had been exposed for 35 days to an antiviral hamster serum of medium potency. The treatment with antiserum, on the basis of preliminary experiments, was expected to decrease the amount of infectious virus adsorbed to the cells.

In each case, the culture was dispersed by trypsin; the cells of the resulting cell suspension were distributed, under a binocular microscope,[3] into drops of medium kept under paraffin oil. Exactly ten cells were placed into each drop. Two determinations were made in each case. In the first, immediately after distribution (= zero point), the contents of each drop, including the cells, were transferred to individual tubes containing 0.5 ml of medium. In the second determination, the drops were incubated for four days before being transferred to individual tubes. At the end of this period, there was an average of 8 cells per drop in the experiment done before the antiserum treatment and an average of 45 cells per drop in the experiment done after the antiserum treatment, with little variation from one drop to another (for accidental reasons, the conditions in the first experiment were unfavorable for cell multiplication). One-fifth of the contents of each tube in both tests was assayed for PY virus after three cycles of freezing-thawing. Tubes having no or little virus were subsequently assayed in their entirety by using 3 to 4 plates for each tube.

Before considering the results of these experiments, it should be recalled that in both experiments the zero point gives the distribution of mature virus present in association with the cells at the start of the experiment; this virus represents the sum of superficially adsorbed virus and of progeny virus that has not yet been released. The four-day count gives a distribution which corresponds to the sum of the zero-point distribution plus that of the virus produced by the cells in the four-day period.

The results of the two experiments, given in Table 1, show that the four-day distributions can be divided into two parts (which are separated by a vertical line in the table). In each case, the left part is equivalent to the zero-point distribution; the right part is characterized by larger amounts of virus in the drops. Since these larger amounts are absent in the zero-point distributions, most of them must represent yields of progeny virus produced during the four-day period. The right parts of the four-day distributions are similar in the experiments before and after antiserum; in contrast, the zero point distribution and the left part of the four-day distribution show a great reduction in the amount of virus in the antiserum-treated culture (compare, for instance, the zero classes). This result suggests very strongly that the zero-point distributions and the left part of the four-day distributions mostly represent virus superficially adsorbed to the cells.

The data of Table 1 show that larger yields were present in 12 out of 75 drops collected after four days in the experiment before the antiserum treatment, and in 32 out of 95 drops collected after four days in the experiment after antiserum. Since the large yields were present in a minority of the drops, most of them must have been produced by single cells. Therefore, as a first approximation, 12 out of 750 (1.6%) initial cells yielded progeny virus in the experiment before antiserum,

TABLE 1

DISTRIBUTION OF VIRUS IN DROPS OF MEDIUM SEEDED WITH TEN CELLS OF LINE 146

Experimental point	Plaque-forming units per drop: 0	1–10	11–50	51–100	101–150	151–200	201–250	251–300	301–350	351–400	401–450	451–500	501–550	551–600	601–650	651–700	>700	Number of drops
Before antiserum																		
Zero point	0	10	9	4	2	..	..	..	..	..	..	..	..	..	..	..	..	25
4-day determination	1	35	15	10	2	3	4	..	2	..	..	..	..	..	1	..	2	75
After antiserum																		
Zero point	31	14	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	45
4-day determination	50	13	5	9	5	1	4	..	3	2	..	..	2	..	..	..	1	95

In the experiment before antiserum, 4740 PFU of progeny virus (right part of the 4-day distribution) were produced by 12 cells, with an average yield of 395 PFU per cell; if calculated on the basis of the 750 cells used in the experiment, there was an average virus production of 1.6 PFU per cell per day. In the experiment after antiserum, 6614 PFU were produced by 32 cells, with an average yield of 207 PFU per cell. The average production, on the basis of the 950 initial cells, was 1.7 PFU per cell per day.

and 32 out of 950 (3.3%) initial cells did so in the experiment after antiserum; the average yield per virus-producing cell was 395 and 207 PFU, respectively.

That virus production was normal in the drops is seen by the similarity of the yield per cell, averaged over all the cells, in the drop experiments and in a comparable mass culture. In fact, the average yield was about one PFU per cell per day in the drop experiments (see Table 1) and in the mass culture.

Properties of clones derived from the virus-releasing mouse embryo line 146: To determine whether the property of releasing virus is transmitted hereditarily, two cloning experiments were done. In each experiment, a culture of the subline 146(3), which was kept under antiviral serum and on which the distribution of the previous section was carried out, was dispersed with trypsin; single cells were transferred under microscopic observation to petri dishes containing as feeder layers secondary mouse embryo cultures which had been irradiated with 5000 r on the previous day. To avoid viral or cellular cross-contamination of the clones, only one cell was transferred to each feeder plate. In all, 200 cells were distributed. Of these, 43 were lost due to contamination or detachment of the cell layer; of the remaining 157 cells, 82 developed into clones; the cloning efficiency was therefore 56 per cent. Each clone gave rise to an actively growing culture.

The clonal cultures were kept under observation for one month in experiment 192 and for three months in experiment 198; during this period they were repeatedly tested for the presence of PY virus. It was found that 53 per cent of the clones in experiment 192 and 51 per cent in experiment 198 never released virus, whereas the remaining cultures released virus at a rate comparable to that of the parental culture. The observed proportion of clones not releasing virus is a minimal value, since clones not releasing virus could have become superinfected (see later) as a consequence of virus multiplication in the feeder layer; in fact, infection of the susceptible feeder layer by virus introduced with the cell is to be expected in some cases. From the point of view of morphology or growth characteristics, the clonal cultures not-releasing virus could not be differentiated from the cultures releasing virus. Thus, although the transformed mouse embryo culture obtained after infection with PY virus and kept as mass culture in the presence of antiviral serum

for a month continued to release virus, it contained many cells which on cloning gave rise to cultures not releasing virus.

To test whether the simultaneous presence, in the mass culture, of cells releasing virus and of cells giving rise to clones not releasing virus was a consequence of a heterogeneity of the culture before infection, a clonal culture releasing virus was in turn cloned after a brief exposure to antiviral serum. Again, the majority of these second-generation clones did not release virus, thus showing that the difference in the properties of the cells to release virus are not due to a preexisting heterogeneity.

The isolation of clones not releasing virus from the virus-releasing mass culture explains the finding that tumors induced in mice by PY virus may cease to produce virus upon transplantation.[4, 5]

Superinfectibility by PY virus of clonal cultures not releasing virus: The results described in the previous sections show that the majority of the cells in the virus-releasing mass culture do not release virus and do not transmit the potentiality for spontaneous virus release to their progeny. This raised the question of whether the persistent virus release in the mass culture was due to the continued reinfection of a small proportion of the cells.

Superinfection experiments were therefore carried out with five of the clonal mouse embryo cultures not releasing virus from experiment 198. Subcultures of these clonal lines, when infected with either *sp* or *lp* virus became persistent virus-releasers, the amount of virus released being similar to that of the parental mass culture. The study of the distribution of virus yields per cell over a 4-day period, carried out in a drop experiment similar to the one described in a previous section, showed again that only a few per cent of the cells became virus yielders. Furthermore, the cultures which had been superinfected with *lp* virus became releasers of *sp* virus 20 to 30 days after superinfection. Thus the superinfected clonal cultures became indistinguishable from the parental virus-releasing mass culture.

It should be remarked that the clonal cultures are considerably more resistant to PY virus than normal mouse embryo cultures. In fact, when superinfected, they show not only a small proportion of cells releasing virus, but also an unaltered overall growth rate and the absence of noticeable cell degeneration.

Conclusions.—The present results show that the persistent virus release, which is present in some of the cultures transformed by PY virus and absent in others, is a superimposed phenomenon. The neoplastic cells arising as a consequence of virus infection in either mouse or hamster embryo cultures are intrinsically nonvirus-releasers. Persistent virus release is caused and maintained in the mass cultures by reinfection of a small proportion of the cells. In this persistent infection, the *sp* mutant of PY virus, presumably preexisting in *lp* stocks, ultimately predominates; this mutant, which is more cytocidal than the *lp*-type virus on normal sensitive mouse embryo cells,[6] has a selective advantage in cultures of neoplastic cells.

An essential factor in the phenomenon of persistent virus release is the increased resistance of the neoplastic cells to superinfection by PY virus. In most transformed hamster cultures, the resistance is so high that no or very few virus-releasing cells arise upon superinfection; in transformed mouse embryo cultures, the resistance is weaker, and a few per cent of virus-yielding cells can be produced. This small proportion of virus-yielding cells is sufficient to maintain virus release in-

definitely in a state comparable to the "carrier state" obtained in many other animal cell-virus systems.[7, 8]

The mechanism by which the persistent release of *sp* virus arises seems completely distinct from the mechanism by which the PY virus causes the neoplastic transformation. For this reason, the mode of production of *sp* virus does not illuminate the mechanism of neoplastic transformation.

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[1] Vogt, M., and R. Dulbecco, these PROCEEDINGS, **46**, 365–370 (1960).

[2] Medina, D., and L. Sachs, *Virology*, **10**, 387–388 (1960).

[3] Lwoff, A., R. Dulbecco, M. Vogt, and M. Lwoff, *Virology*, **1**, 128–139 (1955).

[4] Sachs, L., and M. Fogel, *Virology*, **11**, 722–736 (1960).

[5] Habel, K., Nat. Canc. Inst. Monograph No. 4, 207 (1960).

[6] Gotlieb-Stematsky, T., and S. Leventon, in press.

[7] Vogt, M., and R. Dulbecco, *Virology*, **5**, 425–434 (1958).

[8] Puck, Th. T., and S. J. Cieciura, in *Symposium on Latency and Masking in Viral and Rickettsial Infections*, eds. Duard L. Walker *et al.* (Burgess, 1957).

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*THE SPARING EFFECT OF RMC POLIOVIRUS ON PRIMARY AMNION CELL CULTURES**

BY MONTO HO

DEPARTMENT OF EPIDEMIOLOGY AND MICROBIOLOGY,
GRADUATE SCHOOL OF PUBLIC HEALTH, UNIVERSITY OF PITTSBURGH

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In previous communications,[1, 2] one mechanism whereby cell cultures persist in the presence of an ordinarily cytopathogenic virus was described. Viral inhibitory factor (VIF), probably a variety of interferon,[3] was shown to account for the diminished cytopathic effect (CPE) of a Type II MEF_1 poliovirus variant adapted to the chick embryo (RMC virus[1, 4]) in human amnion cultures receiving undiluted inocula as compared to the complete CPE in those that received diluted inocula. However, it was pointed out[2] that VIF could *not* explain all forms of resistance of amnion cultures to RMC virus, such as that of amnion cells of young *in vitro* age.[5] Further studies of certain resistant cultures have shown that not only is initial CPE overcome but their prolonged survival may be favored.

Materials and Methods.—Primary amnion cultures were prepared as previously described[2] except that instead of Enders' medium containing 5 per cent horse serum, 5 per cent beef embryo extract, 45 per cent bovine amniotic fluid, and 45 per cent Hank's balanced salt solution (BSS), Eagle's basal medium in BSS modified to contain 10 per cent horse serum and antibiotics was used. Cells were grown on the glass surface of 16 mm test tubes containing 1 ml medium. RMC virus used in these studies was from a stock chick embryo passage or from an amnion culture passage. The latter was collected from cultures of amnion cells in Roux bottles two days after stock RMC virus at input multiplicity of about 1 was inoculated. Such harvests also contained VIF.[2]