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Behavior of Tissue Culture Cells Infected with Polyoma Virus*

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Abstract. Infection of tissue culture cells with the oncogenic polyoma virus, or its temperature-sensitive mutant Ts-a, causes several changes other than the previously known induction of cellular DNA synthesis. Cellular movement, survival, and mitotic rate are enhanced in low-serum medium, and morphology is changed; the cellular growth parameters, wound serum requirement, and topoinhibition are markedly decreased. The changes are similar to those that occur in cell transformation and are produced by viral functions known to be expressed in transformed cells. Clues as to the possible mechanisms of all these changes are analyzed and a possible mechanism is discussed.

Infection of tissue culture cells with an oncogenic virus, polyoma or SV40, has two different sets of consequences.[1] When the cell–virus system is nonproductive, i.e., there is no viral multiplication (because either the cells are not permissive or the virus is a nonproductive mutant), either abortive[2] or stable transformation may result. When the system is productive and the cells are in a resting state, the synthesis of cellular DNA and of cellular enzymes related to DNA synthesis are induced.[3] Suitable experimental conditions, however, allow detection of other consequences of virus infection, which help in understanding the significance of the induction of cellular DNA and enzyme synthesis, such as cellular growth parameters[4] and, in serum-starved cells, cellular morphology, movement, survival, and mitotic rate.

Materials and Methods. The virus used was either LP polyoma virus[5] or its temperature-sensitive mutant, Ts-a.[6] Either clarified supernatants of infected cultures or virus purified by equilibrium sedimentation in CsCl was employed as virus stock. Both preparations had identical effects, showing that nonviral substances present in unpurified stocks play no role in the effects described. Infections were carried out at a multiplicity of about 10 plaque-forming units per cell.

The cells employed were derivatives of either the 3T3[7] or the BALB/c-3T3[8] lines. All cultures were in Nunc plastic dishes (4-Shore, U.S.A., P.O. Box 264, La Jolla, Calif.) 50 mm in diameter, with reinforced Eagle's medium (REM) supplemented with 10% calf serum. Secondary mouse embryo cultures were also employed, in REM with 10% horse serum. The cultures were kept in incubators containing a suitable CO_2–air mixtures at 37°C unless otherwise specified. The conditions for infection, wounding, fixing, staining, labeling, and radioautography were described.[9]

DNA synthesis was measured after labeling with [^{3}H]thymidine, either as total synthesis per culture by the Hirt extraction,[10] or by autoradiography.

Measurements of movement: The methods, which allow statistically-significant determinations based on large numbers of cells, use the migration of cells into a

wound. *Method A.* Wounds of a nearly constant width were produced with the same bent-glass rod (about 1 mm in diameter) for all the cultures of a set. The width of the wound was determined by microscopic observation of cultures fixed and stained before movement began, but after cells dislodged during wounding had spread on the plastic (4–6 hr); a standard ocular grid micrometer and standard optics were used. The movement of cells from the edges into the wound was observed in cultures fixed and stained at various times by counting the numbers of cells within the wound in a number of microscopic fields. A difficulty in these experiments is that a uniform wound width is difficult to achieve. *Method B* is independent of wound width and determines the changes in the gradient of cell concentration at either edge of the wound which are the consequences of cell movement (Fig. 1). During these changes, the cell concentration around

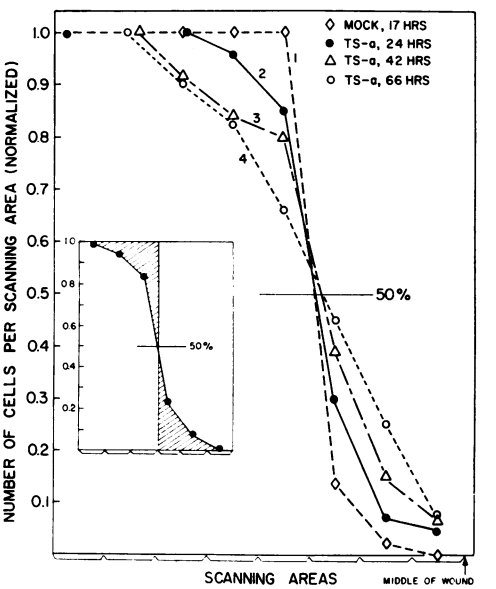

FIG. 1. Cell concentration gradients at a wound edge, determined as cell movement proceeds from minimum (*curve 1*) to maximum (curve *4*). The inset shows the area measured (*cross-hatched area*) for determining the extent of movement.

either edge of the wound follows lines whose slopes decrease with time and which pivot approximately around the 50% value (taking the concentration in the undisturbed layer as 100%). The gradient of cell concentration at a given time is determined by counting cells in thin rectangular areas with the longer sides parallel to the axis of the wound, using an ocular grid micrometer. Each set of rectangles scans across the wound from the intact cell layer on one side of it to that on the other side. When the gradient is determined, the movement is calculated as the total crosshatched area in the inset of Fig. 1. The value obtained is normalized to the cell concentration in a rectangular area in the layer far from the wound.

With either method, only values obtained using the same micrometer and microscopic optics are comparable with each other. Data reported in this article were not obtained with uniform optics, and they are not necessarily comparable with each other. However, data within each experiment are comparable. Each movement value is based on counts of at least 500 cells; therefore differences of 15% are significant.

Cell survival was measured, from the concentration of attached cells (at various times) per microscopic field, using a 100× oil immersion objective. Each point is based on counts of several hundred cells.

Mitotic rate was measured by counting mitotic cells (excluding prophases), as well as total cells, in many microscopic fields.

Cell growth parameters were determined as described.[4]

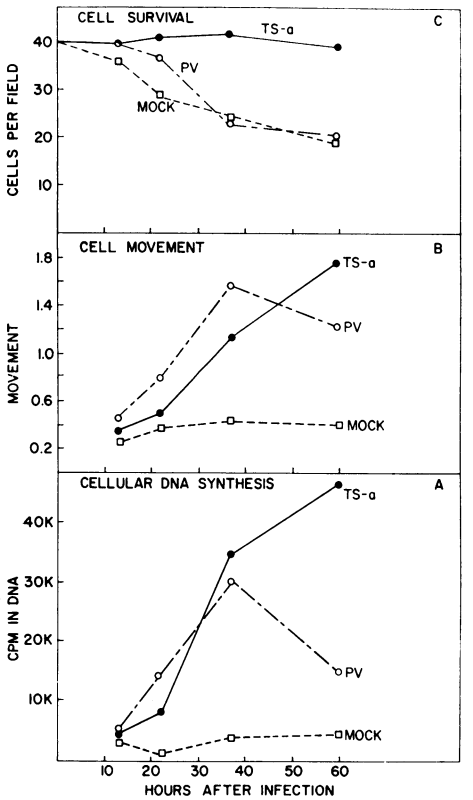

FIG. 2. Effect of infection on cellular DNA synthesis, cellular movement, and survival. Confluent cultures of BALB/c-3T3 cells were washed with REM, incubated for 12 hr in REM, freed of medium, and either infected with virus, or mock-infected. They were then overlaid with 5 ml of REM containing 0.15% horse serum. The cultures were incubated at 39°C and processed 40 hr after infection. Cellular DNA synthesis was determined after fractionation by the Hirt procedure. Cellular movement was determined by *Method B*, and cell survival by counting the cells that remained attached to the dishes.

Results. Induction of movement: In the experiment of Fig. 2, induction of movement was determined together with induction of cellular DNA synthesis. The results (Fig. 2*A*,*B*) show that induction of movement occurs simultaneously with the induction of host DNA synthesis, and follows the same time course. The curves obtained with wild type virus decline after reaching a maximum because cells are being killed by infection; the curves obtained with Ts-a virus do not show a decline, owing to absence of cytopathic effect at the temperature employed.

Similar results were obtained in several other experiments with either 3T3 or BALB/c-3T3 cells. In 3T3 cells, however, the correlation between induction of DNA synthesis and of movement is not complete because I have observed induction of movement but not of DNA synthesis in the absence of serum; in the presence of 0.2% serum, both processes initiated at the same time (Table 1).

Cell survival: BALB/c-3T3 cell cultures accumulate dead cells and lose attached cells at suboptimal serum concentrations.[4] The effect is very noticeable at 39°C, but almost absent at 31.5°C (Fig. 3), and presumably derives from the thermal denaturation of a labile cellular component that is prevented by the serum. The effect of infection on cell survival is shown in Fig. 2*C*: Ts-a infection completely protects the cells; infection with wild type virus seems not to protect, but this result is due to the cytopathic effect of the virus.

TABLE 1. *Relationship between movement and induction of cellular DNA synthesis in cultures of 3T3 cells.*

Hours after infection	Virus	(A) 0.2% serum Movement	(A) 0.2% serum DNA synthesis cpm	(B) No serum Movement	(B) No serum DNA synthesis cpm
9	PV	0.38	65	0.37	25
	Mock	0.39	60	0.41	29
22	PV	1.00	279	1.11	35
	Mock	0.77	73	0.83	59
27	PV	1.27	3,388	0.92	74
	Mock	0.79	114	0.66	37
36	PV	...	10,913	0.67	27
	Mock	...	115	0.51	34

(*A*) Confluent 3T3 cultures in 2% serum were freed of medium, then infected with wild type polyoma virus or mock infected. They were then overlaid with 0.2% calf serum in REM. (*B*) Cultures were washed with REM, covered with REM for 24 hr, freed of medium, and infected. They were then washed with REM and covered with 5 ml REM. In both procedures, the cultures were wounded after infection, and some were labeled with [^{3}H]thymidine, 2 μCi/ml, 1 μM. Movement was determined by *Method B* (see text). Incorporation of label into cellular DNA was measured after Hirt extraction; $^1/_{20}$ of each pellet was counted. Counts are not corrected for background. The decrease in movement without serum after 22 hr is due to loss of cells, especially in the wound. PV = wild type polyoma virus; Mock = mock-infected.

Mitotic rate: Crowded cultures of BALB/c-3T3 cells were either infected with Ts-a virus or mock-infected, then overlaid with medium containing 10% calf serum. At various times, cultures were fixed and stained and mitotic rates were determined. The results (Fig. 4) show that mitoses soon disappear in the mock-infected cultures, whereas they persist in those infected by virus. The difference of mitotic rates is reflected in the total cell counts. The behavior of

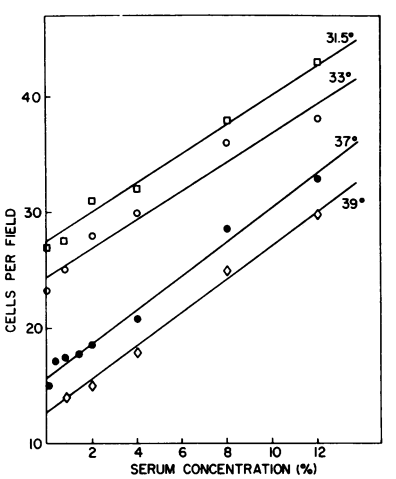

FIG. 3. Effect of temperature on cell survival at various concentrations. Confluent BALB/c-3T3 cultures were washed with REM and overlaid with REM containing various amounts of calf serum. The cultures were divided into groups, each of which was incubated at a different temperature. They were fixed 38 hr later.

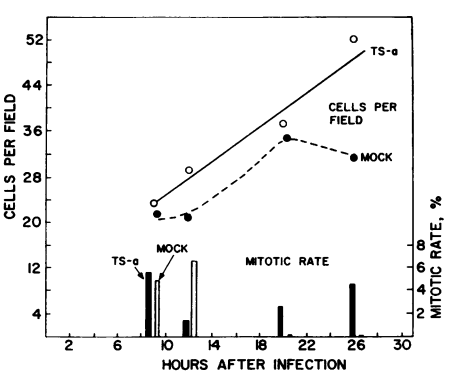

FIG. 4. Confluent BALB/c-3T3 cultures were freed of medium, either infected with Ts-a virus or mock-infected, overlaid with 10% calf serum in REM, and incubated at 39°C. At various times, cells were fixed and stained.

mock-infected cultures can be attributed to serum exhaustion, to which BALB/c-3T3 cells are highly sensitive.

The described effects of infection on both the mitotic rate and survival were repeatedly observed.

Growth parameters: The ability of tissue culture cells to initiate DNA synthesis is affected by many conditions, but mainly by serum concentration and topographical factors.[4] In cultures of BALB/c-3T3 cells, it is markedly temperature dependent (Fig. 5), the proportion of incorporating cells within the

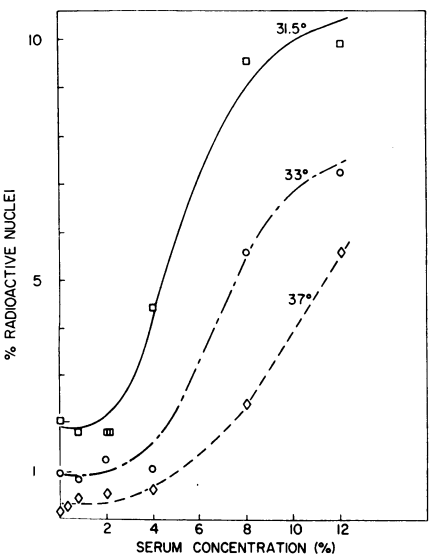

FIG. 5. Effect of temperature on incorporation of [^{3}H]thymidine into DNA of crowded BALB/c-3T3 cultures at various temperatures. The conditions were the same as in the experiment of Fig. 3. The cultures were labeled with [^{3}H]thymidine (2 μCi/ml, 1 μM) 12 hr after changing the medium, and fixed 26 hr later.

same labeling time being paradoxically higher at the lowest temperatures. The explanation may be similar to that invoked for the temperature effect on survival.

Infection of BALB/c-3T3 cells with wild type virus markedly decreases the wound serum requirement and the topoinhibition of the cells.[4] Table 2 shows that both parameters decreased after infection from the high values characteristic of BALB/c-3T3 cells to the much lower values typical of transformed

TABLE 2. *Cell growth parameters of BALB/c-3T3 cells infected by polyoma virus.*

	Virus	Wound serum requirement	Topoinhibition
Expt. *A*	PV	0.05	0.03
	Mock	0.98	0.95
Expt. *B*	PV	0.15	0.28
	Ts-a	0.13	0.40
	Mock	0.95	0.90

(Expt. *A*) Confluent cultures in 10% fetal calf serum in REM were freed of medium, infected with 0.15 ml of virus, washed with REM, covered with 5 ml of REM containing various concentrations of fetal calf serum, and wounded. They were incubated at 39°C; 6 hr later they were labeled with [^{3}H]thymidine (2 μCi/ml, 0.7 μM) and returned to the incubator. They were fixed 40 hr after infection. In Expt. *B*, the confluent cultures were washed with REM and incubated 12 hr in REM. They were infected as above, overlaid with 5 ml of REM containing 0.15% horse serum, wounded, and labeled as above. They were fixed 40 hr after infection. PV = wild type polyoma virus; Mock = mock-infected.

cells, especially in Expt. *A*. In Expt. *B*, the somewhat lower reduction may be attributed to the starvation period.

In secondary cultures of mouse embryo cells kept in 0.5% serum medium for 4 days in order to decrease background DNA synthesis,[11] the infection with wild type polyoma virus caused only a decrease in topoinhibition.

Cell morphology: In serum-starved 3T3 or BALB/c-3T3 cultures, the cells appear very flat, roundish, and faintly stained. After virus infection, they are rather thick, elongated or starlike, and stain more effectively. The morphology of the infected cells is therefore more similar to that of transformed cells.

Discussion. Infection with polyoma virus, either of the wild type or of the Ts-a mutant type, markedly affects cells maintained at low serum concentration, conferring to them attributes that the cells normally have only at high serum concentrations. Several properties are involved, not obviously related to each other: cellular DNA synthesis, movement, survival, mitotic rate, morphology, and growth parameters (e.g., serum requirement for initiation of DNA synthesis in wound cells, and topoinhibition).[4] These effects are recognized especially in cell lines that have extremely high serum requirements, such as 3T3 or BALB/c-3T3; the viral infection makes them much less dependent on serum.

The significance of this replacement can be looked at in the following way. Two cellular properties that were examined for temperature dependence (i.e., survival and initiation of DNA synthesis) were found markedly (and paradoxically) temperature dependent. This suggests that many cellular functions are damaged, especially in low serum medium, by the denaturation of a thermolabile structure that is stabilized by a serum component. It is possible that such a structure is in the plasma membrane of the cells, where it would be easily accessible even to the largest molecules present in the serum. Infection with polyoma virus would modify this hypothetical labile structure, increasing its stability and eliminating the serum requirement. The effect of this modification would concern many cellular properties at once. A justification for this hypothesis is that the cell surface certainly can play a role in cell morphology, movement, and survival, and in allowing mitosis, if only indirectly; and it may also be a link in the regulatory chain controlling DNA synthesis, as suggested by the existence of topoinhibition. It is not clear, however, whether a single virus-induced change can explain all the consequences of infection. In fact reduction of topoinhibition after infection may occur by a separate mechanism for two reasons: in 3T3 cells without serum, movement can be induced without cellular DNA synthesis; and in BALB/c-3T3 cells, topoinhibition is reduced more by infection than by serum, at least within the concentration ranges so far explored. Infection could thus have at least two effects, one decreasing serum requirement for survival, movement, etc., the other bypassing the source of topoinhibition. The latter effect may be the first step in the induction of cellular DNA synthesis by the virus.

These findings show that induction of cellular DNA synthesis is not an isolated phenomenon, but is a part of a complex of changes that affect many seemingly unrelated aspects of cell economy. It may now be possible to begin to search for the molecular bases of the changes.

The ability of the Ts-a mutant to induce all the effects shows that they are caused by viral "early functions" (i.e., expressed in the absence of viral DNA replication), most or all of which are also expressed in transformed cells.[12] It is likely that the complex of cellular changes occurring after infection of permissive cells (before the cells are killed if wild type virus is used) is analogous to abortive transformation,[2] which has been described in nonpermissive cells. The identification of the viral functions responsible for these cellular changes may therefore increase our understanding of the transformation process itself.

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Abbreviation: REM, reinforced Eagle's medium.

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[1] Dulbecco, R., *Science*, **166**, 962 (1969).

[2] Stoker, M., *Nature*, **218**, 234 (1968); Stoker, M., and R. Dulbecco, *Nature*, **223**, 397 (1969).

[3] Dulbecco, R., *J. Amer. Med. Ass.*, **190**, 721 (1964); Dulbecco, R., L. H. Hartwell, and M. Vogt, *Proc. Nat. Acad. Sci. USA*, **53**, 403 (1965); Winocour, E., A. M. Kaye, and V. Stollar, *Virology*, **27**, 156 (1965); Weil, R., M. R. Michel, and G. K. Rushmann, *Proc. Nat. Acad. Sci. USA*, **53**, 1468 (1965); Hatanaka, M., and R. Dulbecco, *Proc. Nat. Acad. Sci. USA*, **56**, 736 (1966); Kit, S., R. A. deTorres, D. R. Dubbs, and M. L. Salvi, *J. Virol.*, **1**, 738 (1967); Branton, P. E., and R. Sheinin, *Virology*, **36**, 652 (1968).

[4] Dulbecco, R., *Nature*, in press.

[5] Vogt, M., and R. Dulbecco, *Virology*, **16**, 41 (1962).

[6] Fried, M., *Virology*, **40**, 605 (1970).

[7] Todaro, G. J., and H. Green, *J. Cell Biol.*, **17**, 299 (1963).

[8] Todaro, G. J., and S. A. Aronson, *Virology*, **38**, 174 (1969).

[9] Dulbecco, R., and M. G. P. Stoker, *Proc. Nat. Acad. Sci. USA*, **66**, 204 (1970).

[10] Hirt, B., *J. Mol. Biol.*, **26**, 365 (1967).

[11] Fried, M., and J. Pitts, *Virology*, **34**, 761 (1968).

[12] Aloni, Y., E. Winocour, and L. Sachs, *J. Mol. Biol.*, **31**, 415 (1968); Oda, K., and R. Dulbecco, *Proc. Nat. Acad. Sci. USA*, **60**, 525 (1968).