Cyclic Variation in Susceptibility of Balb-c 3T3 Cells to Polyoma Virus

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SUMMARY

The yield of polyoma virus from Balb-c 3T3 cells depends on the stage of the cell-cycle at which they are infected and is at a maximum at, or near, the beginning of G1.

INTRODUCTION

In the preceding paper (Thorne, 1973) it was shown that resting monolayer cultures of Balb-c 3T3 cells gave much lower yields of polyoma virus/cell than actively-dividing cultures. In neither case could the yield be markedly altered by various additions to the growth medium. These results raised the possibility that cellular susceptibility varied during the cell cycle depending on some particular transitory factor(s). This problem has been examined using synchronized 3T3 cell populations from two clones which were tested for susceptibility at various times during the cell cycle.

METHODS

Virus and cell cultures. The origins and methods for cultivation of the virus and cells used were described previously (Thorne, 1973).

Procedure for synchrony experiments. The method described by Terasima & Tolmach (1963) for harvesting cells in mitosis was applied to monolayer cultures derived from the two 3T3 clones of highest (5) and lowest (9) susceptibility previously isolated (Thorne, 1973), grown in 32 oz prescription bottles in Dulbecco’s modification of Eagle’s medium containing 10% calf serum in an atmosphere containing 95% air and 5% CO2. The medium on semi-confluent cultures was agitated to suspend loosely attached cells and replaced with 15 ml warmed medium. After 10 min at 37 °C loosely attached cells were suspended by passing the medium over the monolayer 10 to 15 times with a rapid rotatory movement of the bottle about an axis parallel to its long axis. The medium was removed and the procedure repeated 6 more times with fresh medium, all harvests except the first, which was discarded, being cooled rapidly to 4 °C and pooled. The pooled cells were deposited by centrifuging for 10 min at 200 g, resuspended in a small volume of medium, counted, examined for the presence of mitotic cells and seeded into 50 mm plastic Petri dishes at $1 \times 10^4$ to $1 \times 10^6$ cells/dish in 5 ml of medium. The proportion of mitotic cells was assessed as approximately 50 to 70% in several experiments. Medium containing a lowered level of Ca$^{2+}$ as recommended by Robbins & Scharff (1966) was not satisfactory for this system since although cultures grew adequately the monolayers were easily torn and the harvests contained a high proportion of non-mitotic cells.

Other methods of synchrony used are described in Results.

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Fig. 1. Variation in virus yield from Balb-c 3T3 cells infected with polyoma virus at various times after synchronization by mitotic harvest. Upper Fig., clone 5 (two experiments); lower Fig., clone 9. The vertical bar lengths give relative cell numbers for uninfected cells.

At intervals after attachment of the cells was complete the medium was removed from duplicate cultures and the cells were washed with tris-saline and infected with $2 \times 10^7$ p.f.u. of small-plaque polyoma virus in 0.2 ml tris-saline. After an adsorption period of 30 min at 37 °C the cultures were both washed and re-fed with warm medium, labelled with 5 or 10 μCi [$^3$H]-thymidine (sp. act. 6·7 Ci/m-mol) and incubated at 37 °C. An equivalent sample of the mitotic harvest was incubated in suspension in 0·4 ml serum-free medium with $2 \times 10^7$ p.f.u. for 30 min at 37 °C before plating in 4 ml medium containing radioactive label.

**Virus production.** The yield of virus was determined by the following procedure modified from that described elsewhere (Thorne & Wardle, 1973): 48 h after infection the cells were scraped off into the medium and the suspension centrifuged at 200000 g for 1 h to deposit virus from the cells and medium. The deposit was suspended in 0·7 ml 20 % Triton-X-100 in pH 7·5, 0·2 m-tris-HCl buffer and the suspension incubated successively with RNase (200 μg), DNase (200 μg) in the presence of 0·01 m-MgCl₂, and trypsin (0·1 %) for 30, 30 and 15 min respectively, before analysis for radioactive virus particle yield by equilibrium centrifugation on 2·5 ml CsCl ($\rho = 1·35$ g/ml) for 16 h at 100000 g in the SB-405 rotor of the International B 60 centrifuge.
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RESULTS

Synchronization by mitotic harvest

The yields of radioactive virus obtained from infection of mitotically-harvested cells at increasing times after plating are shown in Fig. 1. The yields varied markedly with time of infection. For clone 5 the yield from cells infected at 2 to 3 h was substantially greater than for cells infected in suspension at time 0. The yield then declined to a minimum at about 11 h, increased again to a maximum at 22 to 25 h and then declined. Limited data suggest the presence of another maximum around 40 h. The number of cells obtained was insufficient to permit full analysis of the cell growth cycle, however, it was determined for uninfected cells that the cell population doubled approximately between 9 and 26 h, i.e. around the period of maximum virus yield, indicating that periods of maximum susceptibility appeared cyclically with a frequency similar to that of cell division. The decline in yield from a time of 2 to 3 h after mitosis indicates that the time of maximum susceptibility occurs near the time of cell division and probably early in G1 phase. Although yields were very much lower the results with clone 9 showed a similar early decline in yield but a relatively much smaller peak around 20 h.

As a control for the above experiments the susceptibility of similarly-harvested non-synchronous clone 5 cells was examined. Cells were dislodged by vigorously pipetting medium over semi-confluent cultures repeatedly to produce tearing. After dispersal to single cells by further pipetting the suspension was plated at $1 \times 10^6$ cells/dish.

In two experiments the yields determined after infection at subsequent intervals (Fig. 2) increased gradually with no pronounced maxima or minima indicating that in sparse
asynchronous populations the yield was largely determined by the number of cells. A similar result was obtained with ‘mitotic’ harvests from cultures maintained in low Ca\(^{2+}\) medium which consisted principally of non-mitotic cells.

**Synchronization by thymidine block**

In an attempt to establish independently the cyclic variation of susceptibility the thymidine block method of synchronization was employed. Cultures in exponential growth 72 h after plating were treated with a double-thymidine block (2.5 mM-thymidine for 15 h with an intervening period of 8 h in thymidine-free medium). After removal of the second block by washing and transfer to thymidine-free medium, duplicate cultures were infected at several subsequent times as described in Methods. The yields were measured by haemagglutinin activity as well as radioactivity since a residual high level of thymidine might have modified subsequent incorporation of \(^{\text{[H]}}\)-thymidine. However, both methods showed (Fig. 3) a maximum at about 2 h after removing the block, i.e. from the beginning of S phase. There was no maximum at this time for cultures of the same age not treated with thymidine but for which the medium was renewed at the time of removal of the block. Since the length of the S phase in 3T3 cells is about 7 h (Nilausen & Green, 1965) this result is consistent with peak susceptibility occurring after the end of S phase.

**Synchronization with colcemid**

To permit further examination of the relationship between the time of DNA synthesis, cell division and the time of maximum susceptibility, for which larger numbers of synchronized cells were required, the use of the colcemid method for arresting cells in mitosis was investigated. The loose cells on semi-confluent monolayer cultures were removed by shaking into the medium, the monolayers washed and the medium replaced with fresh medium containing 0.2 µg/ml colcemid. After 3 h the mitotic cells were removed by shaking,
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Fig. 4. Variation in virus yield from Balb-c 3T3 clone 5 cells infected with polyoma virus at various times after synchronization by treatment with 0.2 μg/ml colcemid for 3 h before mitotic harvest. For the measurement of DNA synthesis the TCA-precipitable radioactivity from cultures pulsed for 30 min with [3H]-thymidine was collected by filtration, dried and dissolved in Hyamine before addition of scintillation fluid and counting. O—O, Radioactive virus yield; O—O, number of cells/plate; ———, DNA synthesis.

collected and washed once with medium by centrifuging and replated in Petri dishes at $3 \times 10^4$ cells/dish. At subsequent times cultures were washed with tris-saline and infected to determine yield of radioactive virus as described in Methods. Uninfected replicate cultures for each time of infection were used to determine cell concentration and others incubated with 5 μCi [3H]-thymidine for 30 min at 37 °C before dissolving the cells in 1 % SDS in NaCl-EDTA to permit estimation of DNA synthesis from TCA-precipitable radioactivity.

The results showed (Fig. 4) two distinct periods during which cell numbers doubled centred around 20 and 42 h. Peaks of DNA synthesis were evident at 25 and 46 h (the period between 3 and 19 h was not investigated).

Thus cells continued to divide after colcemid treatment without abnormal lag and in a substantial degree of synchrony. The yields of virus obtained showed evidence of cyclic variation similar to that observed in cultures synchronized without colcemid; maximum values occurred 8 to 10 h prior to the maxima in cell numbers, and the yield subsequently fell rapidly. It is clear that maximum yields occurred when cell DNA synthesis was low and prior to the main period of DNA synthesis.

In other experiments the period of accumulation of mitotic cells was extended to 15 h using a lower concentration (0.1 μg/ml) of colcemid. In these experiments (Fig. 5) cell number was observed to increase sharply at about 10 h after plating and the single peak of DNA synthesis, indicating a substantial degree of synchrony, reached a maximum at about
Fig. 5. Variation in virus yield from Balb-c 3T3 clone 5 cells infected with polyoma virus at various times after synchronization by treatment with 0.1 μg/ml colcemid for 15 h before mitotic harvest.

○---○, Radioactive virus yield; ○—○, number of cells/plate; ■---■, DNA synthesis.

13 h. However, in two such experiments the virus yields varied in a manner similar to that found for asynchronous cultures. This finding remains to be investigated but it seems probable that prolonged colcemid treatment disturbed normal relationships between cell functions and altered the characteristics of the response to virus infection. Nevertheless, it is again clear that susceptibility remained relatively high when DNA synthesis was low.

DISCUSSION

The restriction of the susceptibility of the 3T3 cells to a limited period in the cell cycle may reflect requirements for the presence or absence of one or more cell components only appearing at specific times. The restriction may be a contributory factor in accounting for the relatively low yield from asynchronous cultures compared to that from mouse embryo cultures and also the progressive decrease in yield as the resting stage is approached and the number of dividing cells decreases (Thorne, 1973).

It seems probable that the susceptible stage is located in the G1 phase but its precise location and relation to other events in the cell cycle has yet to be determined. It is clear that the normal period of DNA synthesis is excluded.

Transient permissiveness for polyoma virus could arise in several ways; e.g. it may represent the cyclic appearance of a specific surface receptor. The cyclic variation of cell surface sites, e.g. blood group antigens (Thomas, 1971), has been established for several cell types. Such a receptor could be newly synthesized or represent a component which becomes un-
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covered for a limited time. Alternatively, susceptibility may be determined by other cellular functions which may be necessary for the initiation of infection, e.g. pinocytotic activity, uncoating enzyme, transcriptase or enzymes affecting integration of virus DNA into the cell genome.

The relevance of the results with 3T3 cells to polyoma virus infection of other cells is unknown. However, the variability in virus yield from primary and secondary mouse embryo cultures commonly encountered could be explained on a similar basis as could the low transformation rates observed in non-lytic systems.

Finally, restriction of susceptibility to a period early in G1 could be related to the time required for initiation of virus and cellular DNA synthesis after infection (14 to 18 h). Productive infection may depend on an interlocking of the initial phase of virus replication with a particular cell programme leading to DNA synthesis on which virus DNA synthesis is dependent.

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REFERENCES


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