Inhibition by Interferon of Polyoma Virus-induced Cell DNA Synthesis in Mouse Peritoneal Macrophages

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SUMMARY

Mouse peritoneal macrophages from C57 Bl and NIH mice were examined after infection with polyoma virus. Cell DNA synthesis was stimulated in both cell types to the same extent between two and three days after addition of virus. Morphological changes appearing soon after infection were reversed by the second or third day. The cells did not acquire other properties associated with the transformed state. Treatment with mouse interferon up to 48 h after infection inhibited the virus-induced host DNA synthesis while morphological changes were not affected.

INTRODUCTION

Two kinds of responses have been noted in cells infected with oncogenic DNA viruses; a lytic interaction, leading to virus multiplication and cell death, and a moderate one involving no vegetative growth of virus but leading to stable transformation of a fraction of the cell population. Transformed cells can then be distinguished from normal cells by several criteria (see reviews by Black (1968) and by Meyer (1971)). Although only a small proportion of the cells becomes stably transformed in the moderate non-permissive systems, the majority of them show some transient changes characteristic of the transformed state. Thus in human diploid cells infected with simian virus 40 (SV 40) (Sauer & Defendi, 1966) and in BHK 21 cells infected with polyoma virus (PV) (Stoker, 1968; Taylor-Papadimitriou, Stoker & Riddle, 1971), T antigen is synthesized by the majority of the cells and synthesis of cell DNA is stimulated and followed by mitosis and cell division under conditions which do not allow growth of untransformed cells.

The most unambiguous way to determine whether the transient changes induced in the non-permissive systems depend on reading of the virus genome is to obtain temperature-sensitive mutants which do not induce these modifications (Fried, 1965; Di Mayorca et al. 1969; Eckhart, 1969; Stoker & Dulbecco, 1969). Another approach is to see if the induced changes can be inhibited by interferon which blocks the expression of virus functions (Isaaec & Lindenmann, 1957; Colby & Morgan, 1971). It has been shown that T antigen synthesis is inhibited by mouse interferon in 3T3 cells infected with SV 40 (Oxman & Black, 1966), although virus-induced cell DNA synthesis is reported to be unaffected (Todaro & Green, 1967). However, hamster interferon is able to inhibit induction of DNA synthesis and abortive transformation in polyoma-infected BHK 21 cells (Taylor-Papadimitriou &
Stoker, 1971). Interferon also inhibits the stimulation of cell DNA synthesis in 3T3 cells productively infected with polyoma virus (Dulbecco & Johnson, 1970). In these systems then there is some evidence to suggest that reading of the virus genome occurs in most of the cells.

The response of mouse peritoneal macrophages to infection with polyoma virus is different from other non-permissive systems in that while incorporation of thymidine into host DNA is stimulated, this is not followed by cell division or stable transformation (Mallucci, 1969). It is therefore relevant to ask the question whether the virus genome is expressed in these cells. The fact that with macrophages from one of the strains used (C57 Bl) T-antigen is also induced would suggest that at least part of the virus genome is expressed.

In this paper we report a more detailed investigation into the properties of polyoma virus infected peritoneal macrophages from both C57 Bl and NIH strains and on the inhibitory effect of interferon on the virus-induced DNA synthesis.

METHODS

**Virus.** A small plaque variant of polyoma virus was grown in confluent cultures of 10-day-old T.O. mouse embryo fibroblasts (Imperial Cancer Research Fund, London) using Dulbecco-modified Eagle's medium supplemented with 5% horse serum. Cultures showing advanced c.p.e. after 8 to 10 days were harvested and virus was extracted and purified as described by Crawford (1969). After dialysis against 0.01 M-tris, pH 7.3, band purified stock virus was suspended in this buffer containing 0.001% bovine serum albumin and stored at -70 °C in measured amounts. Infectivities were measured by plaque formation on secondary embryo cultures as described by Takemoto, Kirschstein & Habel (1966) where bovine serum was replaced with horse serum at a concentration of 5%.

**Cells.** Macrophages were derived from two different strains of mice, C57 Bl and NIH albino strains. Cells were collected without previous peritoneal stimulation and cultured in 199 medium containing 20% inactivated calf serum plus 10 iu heparin/ml using chambers made with 1 cm diam. glass rings mounted on microscope slides with 1:1 melted paraffin and wax mixture. The cultures, each consisting of about 10^5 cells, were kept in humidified Petri dishes and incubated at 37 °C in a current of 5% CO₂. At 3 h after seeding, the medium with the unattached cells was replaced with fresh medium containing no heparin. The cells were infected the following day with 0.1 ml of virus suspension containing 100 p.f.u./cell. After absorption for 2 h at 37 °C the cells were washed and the medium which had been removed before the infection was returned or, in other instances, fresh medium was added.

**Interferon preparations and assays.** Interferon formation was induced in 5-day-old cultures of primary mouse embryo fibroblasts by either u.v.-irradiated Newcastle disease virus (NDV) at a concentration of 200 p.f.u./cell, or by polyinosinic-polycytidylic acid (poly I:C) at a concentration of 10 μg/ml together with 10 μg/ml DEAE-dextran (Field et al. 1968). After incubation with the inducer for 2 h at 37 °C, the cultures were washed twice and incubated in Eagle's modified medium (without serum) for 24 h, at which time the medium was harvested and adjusted to pH 2. After 5 days at 4 °C the interferon preparations were concentrated by absorption to, and elution from, Doucil (J. Crosfield and Sons, Warrington) and finally dialysed against physiological saline (Fantes, O'Neill & Mason, 1964).

Since serum does not stimulate cellular DNA synthesis in macrophages, two preparations of serum interferon from mice infected with u.v.-inactivated influenza PR 8 virus and Newcastle disease virus (kindly supplied by Dr J. Sonnabend and Dr D. Metz), were also used.
Quantitations of interferon activity were carried out as described before in C57 and NIH macrophages (Mallucci, 1969), using mouse hepatitis virus-3 (MHV-3) as challenge virus (Mallucci, 1965, 1966). In accordance with our previous results, both cell strains showed equal sensitivity to the hepatitis virus and in both systems MHV-3 replication was inhibited to a similar extent by the same dose of interferon. An amount of interferon capable of neutralizing $10^4$ f.f.u. (foci forming units) of MHV-3 as judged by c.p.e. was the dose used in all our experiments. This dose corresponded to 500 to 1000 reference research units of mouse interferon. When interferon was added to the cultures before infection, it was removed when the cells were infected, and returned afterwards. In other experiments, interferon was added to the culture medium at different times after infection.

Chick interferon and inactive mouse interferon were used for control experiments. The chick interferon was a gift from Dr Ian Kerr and Dr K. H. Fantes. It consisted of a preparation partially purified on DEAE cellulose (Fantes, 1967). An equivalent of 50 units, assayed by using Semliki Forest virus in chick fibroblasts (Mecs et al. 1967), was added to each macrophage culture.

Inactive mouse interferon was obtained by exposing samples of the interferon preparations to a temperature of 80 °C for 30 min (Glasgow & Habel, 1962). Loss of interferon activity was assessed in cultures infected with MHV-3 virus.

Radioactive isotope labelling. Synthesis of DNA was measured by exposing duplicate cultures to $[^3H]$-methyl-thymidine (5°Ci/m-mol, 5μCi/ml) for 24 h at 24 h intervals. At the end of the labelling period the glass rings were removed leaving the macrophage cultures on the slide. The cells were then washed in phosphate-buffered saline, fixed with formol-saline, washed in xylene, passed through ethanol, washed three times in cold 5% TCA, and washed again in distilled water and in ethanol. The dried cultures were cut out from the slides and transferred with the cells on the upper surface to counting vials to be counted in a liquid scintillation counter.

Cultures showing $[^3H]$-thymidine uptake above background levels were subsequently recovered and stained in order to assess the cell number.

Tests for assessment of the transformed state

Agglutination with and binding of concanavalin A. A significant property of cells transformed by polyoma virus is their ability to agglutinate in the presence of some plant lectins (Burger, 1969; Inbar & Sachs, 1969). Studies with temperature sensitive virus mutants have shown that this property is related to the expression of a specific site of the virus genome (Eckhart, Dulbecco & Burger, 1971). Binding of the plant lectin concanavalin A (Con A) to the cell surface was detected by immunofluorescence using hamster anti-Con A serum and fluorescein conjugated anti-hamster globulin (Progressive Lab. Inc., Baltimore, U.S.A.). Cell agglutination to Con A was scored on cell cultures on coverslips. Both methods have been described in detail in a previous paper (Mallucci, 1971).

Coat thickness. Increase of cell coat thickness is a constant feature of cells transformed by polyoma virus (Martinez-Palomo, 1970). An automatic photoelectric ellipsometer (Poste, 1970) was used to assess the thickness of the cell-surface material left on ellipsometric slides after cell removal by means of freezing and thawing. This method, which has been suitably used to detect cell coat difference between macrophages and other cells (Mallucci, 1971) was adopted, as macrophages, unlike other cells, cannot be detached from the glass by chelating agents.

Growth in soft agar. Colony formation in soft agar was carried out as described by Macpherson & Montagnier (1964) using cells infected in suspension.
Table 1. Tests for transformation properties

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Con A binding (immunofluorescence positive or negative)</th>
<th>Agglutination by con A (positive or negative)</th>
<th>Cell coat thickness Å ± s.e.</th>
<th>Efficiency of colony formation in soft agar (%)</th>
<th>% tumours induced by 10^6 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal macrophages*</td>
<td>+</td>
<td>--</td>
<td>55 ± 12</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Infected macrophages*</td>
<td>+</td>
<td>--</td>
<td>55 ± 12</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>PV-TT-3</td>
<td>+</td>
<td>+</td>
<td>150 ± 8</td>
<td>63</td>
<td>100</td>
</tr>
<tr>
<td>Secondary fibroblasts</td>
<td>+</td>
<td>--</td>
<td>75 ± 6</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* Tests carried out at day 3, 6, 9 and 14 in infected cells and in control cells of the same age.

Antigenicity. Anti-tumour surface antibodies were raised in rabbits by four injections, each of 10^6 polyoma transformed cells (clone PV-TT-3) which had been detached from the glass with a rubber policeman. Antibody specificity to the tumour cells was assayed by means of [3HNa]-CrO₄ release (Raff, 1969), modified for monolayer cultures, after pre-adsorption of non-specific antibodies to homologous normal cells.

RESULTS

Morphological changes in mouse peritoneal macrophages infected with polyoma virus

Macrophages infected with polyoma virus undergo transient morphological alterations consisting of moderate rounding followed by spreading and a certain degree of vacuolation. These changes, which are more accentuated and persistent in the C57 than in the NIH strain become well evident 24 h post-infection and are followed after 2 to 3 days by a gradual return of the cells to what may appear to be their normal state.

Pre-treatment of cells with interferon had no effect on the appearance and progression of the changes. This observation suggests that the morphological alterations reflected a non-specific response of the cell to invading particles rather than being dependent on the expression of the virus genome. Supporting this idea is the observation that the changes persist only as long as the infecting inoculum can be detected in the cells both as infectious units and as immunofluorescent particles in the cytoplasm.

Absence of transformed properties in polyoma-infected macrophages

Certain properties have been shown to be associated with the transformed state, and parameters related to these properties were measured in polyoma virus infected macrophages at various times after infection. Table 1 shows that changes related to cell surface characteristics and growth control mechanisms observed in a line of C57 Bl PV-transformed cells (PV-TT-3) were not found in macrophages of the same strain up to 14 days after infection. In addition no antibodies to PV-transformed cells from C57 mice could be detected in the serum of C57 mice injected with 5 × 10^6 homologous macrophages which had been infected 14 days before. The data in Table 1 applies to both C57 Bl and NIH cells but in the case of the NIH macrophages a comparison with a corresponding transformed cell line could not be done as polyoma virus does not produce tumours in this strain nor does it induce transformation in vitro (L. Mallucci, unpublished observations). Even after keeping infected macrophages in culture for up to five weeks no signs of transformation could be detected.
Interferon inhibition of PV-induced DNA synthesis

Fig. 1. Effect of pre-treatment with 1000 reference research units of mouse interferon on DNA synthesis in polyoma-infected mouse peritoneal macrophages; ■—■, cultures treated with interferon 16 h before infection; •—•, untreated infected cultures; ○—○, uninfected cultures.

Table 2. Effect of heat-inactivated interferon and of heterologous interferon on the growth of MHV-3 and on the stimulation of DNA synthesis by polyoma virus in mouse peritoneal macrophages

<table>
<thead>
<tr>
<th>Interferon preparations*</th>
<th>NIH macrophages</th>
<th>C57 Bl macrophages</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MHV-3 titre†</td>
<td>ct/min/5×10⁴ cells‡</td>
</tr>
<tr>
<td>Nil</td>
<td>5.0</td>
<td>2500</td>
</tr>
<tr>
<td>Mouse serum interferon (PR-8)</td>
<td>1.5</td>
<td>600</td>
</tr>
<tr>
<td>Heat-treated mouse serum interferon (PR-8)</td>
<td>4.5</td>
<td>2000</td>
</tr>
<tr>
<td>Poly I:C induced interferon in mouse fibroblasts</td>
<td>2.7</td>
<td>800</td>
</tr>
<tr>
<td>Heat-treated poly I:C induced interferon in mouse fibroblasts</td>
<td>4.5</td>
<td>2000</td>
</tr>
<tr>
<td>Chick interferon</td>
<td>4.5</td>
<td>2300</td>
</tr>
</tbody>
</table>

* The same amount of mouse interferon (500 to 1000 reference research units) was used in each case.
† Log₁₀ TCID₅₀/ml produced over a period of 48 h; calculation according to the method of Reed & Muench (1938). (Cultures in test tube; four tubes per dilution).
‡ [³H]-Thymidine, 24 h pulse, from 72 to 96 h post-infection.

Pre-treatment of macrophage cultures with interferon; effect on virus stimulated DNA synthesis

Interferon was added to macrophage cultures from C57 Bl and NIH mice 16 h before infection with polyoma virus as described in Methods and the amount of [³H]-thymidine incorporated into acid-insoluble material over a 24 h period was estimated daily after infection. Fig. 1 shows one such experiment.

The infected cells begin to incorporate labelled thymidine between 2 and 3 days post-infection, the rate becoming maximal by the 4th day. Pre-treatment with interferon effectively
Table 3. Effect of interferon added at various times after infection on polyoma-induced DNA synthesis in mouse macrophages

<table>
<thead>
<tr>
<th>Time when interferon was added (h)</th>
<th>NIH macrophages (ct/min/5 × 10^4 cells)</th>
<th>C57 B1 macrophages (ct/min/5 × 10^4 cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>72 to 96 h</td>
<td>96 to 120 h</td>
</tr>
<tr>
<td>0</td>
<td>180</td>
<td>200</td>
</tr>
<tr>
<td>10</td>
<td>200</td>
<td>200</td>
</tr>
<tr>
<td>24</td>
<td>250</td>
<td>300</td>
</tr>
<tr>
<td>48</td>
<td>550</td>
<td>350</td>
</tr>
<tr>
<td>72</td>
<td>2100</td>
<td>1200</td>
</tr>
<tr>
<td>96</td>
<td>.</td>
<td>.</td>
</tr>
<tr>
<td>No interferon</td>
<td>2200</td>
<td>1200</td>
</tr>
<tr>
<td></td>
<td>2400</td>
<td>1590</td>
</tr>
</tbody>
</table>

Macrophage cultures were infected with virus and treated with interferon as described in methods. [3H]-Thymidine was added for the time indicated and radioactivity incorporated into TCA-insoluble material was estimated.

reduces this virus-stimulated incorporation to little above background values. The same picture was seen with both macrophage strains, and with the same dose of interferon preparations of different origin as stated in Methods.

Preparations of heat-inactivated interferon and a preparation of chick interferon were used in control experiments. Table 2 shows that loss of interferon activity was accompanied by loss of inhibitory effect on the stimulation of DNA synthesis by polyoma virus.

**Effect of interferon added after virus infection on the incorporation of [3H]-thymidine into DNA**

It is assumed that interferon acts on an intracellular event in inhibiting virus functions, but this has not been demonstrated for every virus-cell system used. The long lag (3 days) between virus infection and stimulation of thymidine incorporation in the macrophage system suggested that it might be possible to add interferon after virus penetration and still get an effect. Interferon was therefore added after virus at the time indicated in Table 3 and incorporation measured between 3 and 4 days and between 4 and 5 days. It can be seen that interferon added up to 48 h after the virus is effective in blocking induction of DNA synthesis in the infected macrophages. However, once cell DNA synthesis has started, interferon is no longer capable of exerting an inhibitory effect. This behaviour indicates that interferon is active in preventing the expression of a function required for initiation of cell DNA synthesis.

**DISCUSSION**

Mouse peritoneal macrophages which ordinarily do not synthesize DNA either in vivo or in vitro (Van Furth & Cohn, 1968) represent a system of special interest in investigating the role played by DNA oncogenic viruses in the activation of cellular DNA synthesis and in causing the appearance of other functions associated with the transformed state. Transformation of macrophages by SV40 virus has been reported by Stone & Takemoto (1971) and by Mauel & Defendi (1971). The results presented here indicate that neither NIH nor C57 B1 macrophages, infected with polyoma virus, behave like transformed cells with regard to cell coat thickness, agglutinability by concanavalin A, growth in soft agar, tumour-inducing ability or ability to induce cell surface specific antibodies to PV-transformed homologous cells. Morphological changes are observed before DNA synthesis is induced but interferon has no effect on these changes, indicating that they are not a manifestation
Interferon inhibition of PV-induced DNA synthesis of abortive transformation as is found in polyoma infected BHK-21 cells (Stoker, 1968; Taylor-Papadimitriou & Stoker, 1971). However, the fact that virus-induced DNA synthesis is inhibited by interferon added during the time (48 h) preceding this event but not after, suggests that a virus function required for DNA initiation is being expressed in the infected cells during this period. That interferon can inhibit the activation of DNA synthesis even when added up to two days after infection supports further the idea that it is not entry of virus which is being inhibited but an intracellular function.

The assumption that interferon is inhibiting specifically a virus function may be questioned in the light of recent observations of Lindahl-Magnusson, Leary & Gresser (1972) who reported inhibition of phytohaemagglutinin-stimulated DNA synthesis in mouse lymphocytes by interferon. On the other hand, serum-stimulated DNA synthesis in BHK-21 cells is not inhibited by interferon (Taylor-Papadimitriou & Stoker, 1971). Relevant to this point are the data of Borecký et al. (1972) showing that the antiviral effect of interferon is independent of its cell-inhibitory activity. We have observed that u.v.-inactivated polyoma virus particles do not activate DNA synthesis in either strain of macrophages nor do they induce appearance of T-antigen in the C57 cells (L. Mallucci & J. Taylor-Papadimitriou, unpublished observations), a fact that further suggests that a virus function must be expressed to activate cell-DNA synthesis. The pattern of cell DNA synthesis following polyoma virus infection and its response to interferon treatment is exactly comparable in both strains of macrophages. This indicates that the function required for stimulation of host DNA synthesis is expressed at the same time, in both cell types.

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REFERENCES


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