Transformation of BSC 1 Cells following Chronic Infection with SV40

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(Accepted 24 April 1969)

SUMMARY

The BSC/SV40 virus carrier state was established by infecting monolayers of BSC1 cells with high concentrations of SV40. In these carrier cultures, only 4% of the cells contained intranuclear tumour (T) antigen as determined by the immunofluorescent method, but every cell produced infectious virus. During passage of the BSC/SV40 cultures, a cell line was selected in which all the cells exhibited T antigen and only 0.2–1% of the cells produced infectious virus (transformed state). The BSC1 transformed cells were like the BSC1 parent cells sensitive to challenge with heterologous viruses (attenuated polio I, herpes simplex and vaccinia), but were resistant to superinfection by the homologous SV40. Cytosine arabinoside, actinomycin D and antiserum to SV40 inhibited virus and T antigens in the BSC/SV40 carrier cultures. In the transformed cells, infectious virus and virus antigen only were inhibited but synthesis of the T antigen remained unaffected. By cell cloning, two virus-free clones were obtained.

INTRODUCTION

Propagation of SV40 in primary green monkey kidney or BSC1 cells (Hopps et al. 1963) results in cytopathic changes leading to lysis of the cultures (Hsiung & Gaylord, 1961; Steinbaugh & Melnick, 1962; Kit et al. 1966), but SV40 transforms cells of hamster, rabbit, mouse, bovine and human origin (Black & Rowe, 1963a–c; Jensen, Koprowski & Pontén, 1963; Diderholm et al. 1965; Diderholm, Berg & Wesslén, 1966). Transformation of rhesus muscle, skin and testicle cells has been reported by Rabson, Kirschstein & Legallais (1965) and that of green monkey kidney cells by Fernandes & Moorhead (1965) and Wallace (1967).

By infecting BSC1 cell monolayers with a high multiplicity of SV40, we obtained a chronic infection of the cells (BSC/SV40) with characters similar to other virus carrier states (Walker, 1964). The BSC/SV40 carrier cultures yielded infectious virus and showed the presence of both tumour (Black et al. 1963; Rapp et al. 1964; Habel et al. 1965; Hoggan et al. 1965; Kitahara, Melnick & Rapp, 1966) and virus antigens in a fraction of the cell population. On passage of the BSC/SV40 carrier cultures, infectious virus and virus antigen became undetectable and the percentage of cells containing tumour (T) antigen increased to 100% (transformed state). We report here a quantitative study of infectious virus and virus and T antigens during passage of the BSC/SV40 carrier cultures and present the differences between the virus carrier and the transformed state.
METHODS

Viruses. SV40 was propagated in 4-day-old BSC1 monolayers; SV40 suspensions were stored at -20° and sonic-treated for 90 sec. before use. The original seed came from a plaque-purified SV40 preparation (Ashkenazi & Melnick, 1962). Attenuated poliovirus type 1 was propagated in baboon kidney cells and stored at -20°. Vaccinia virus (NOGUCHI strain) was propagated on the chorioallantoic membranes of 12-day chick embryos and stored at -20°. Herpes simplex virus was propagated in BSC1 monolayers and stored at 4°.

Cells and media. BSC1 cells obtained from Microbiological Associates, Bethesda, Md, U.S.A., were grown in Eagle's basal medium containing four times the concentration of amino acids and vitamins (Eagle's modified) supplemented with 10% calf serum. Hamster kidney cells transformed by SV40, the HK-7 cell line (Ashkenazi, 1965), were grown in Eagle's modified medium supplemented with 10% calf serum. BSC1/SV40 cell monolayers and the transformed BSC1 cells were each split 1:3 and passaged every 5 or 6 days in Eagle's modified medium supplemented with 10% calf serum.

Infectivity titrations. These were made on 4-day-old BSC1 monolayers in tubes or in 60 mm. Falcon plastic Petri dishes. The virus was adsorbed for 3 hr at 37°. The tube cultures were washed with Hanks's solution and inoculated with 0.5 ml. of the appropriate virus dilution. To tube cultures, 2 ml. of Eagle's modified medium supplemented with 2% calf serum were added, and the medium changed every 6 days. The tube monolayers were observed for the characteristic cytopathic effect during a period of 30 days. The monolayers in the Petri dishes, following washing and virus inoculation, were overlaid with 6 ml. overlay which consisted of 3 ml. 2% Difco Noble agar and 3 ml. double-strength modified Eagle's medium supplemented with 10% calf serum. They were incubated in a humidified incubator containing 5% CO₂. On the second and 7th days, 3 ml. of Eagle's modified medium supplemented with 5% calf serum were added, to prevent drying of the overlay. After 16 days of incubation, the monolayers were stained with a mixture of 1 ml. neutral red (1/3,000) and 1 ml. 2% Difco Noble agar. Plaques were visible 10 to 24 hr after staining.

Poliomyelitis, vaccinia and herpes viruses were titrated on monolayers of BSC1 cells in Petri dishes; the virus suspensions were adsorbed for 1 hr and the monolayers overlaid with the overlay used for SV40, supplemented with 3% calf serum. They were stained 4 days after inoculation.

Immunofluorescent technique. The cells were grown in Leighton tubes on coverslips. At different periods, the coverslips were withdrawn, washed three times with Hanks's solution, twice with acetone and fixed with acetone for 3 min. They were then air-dried and stored at -70°. For detection of the T and virus antigens, the coverslips were stained for 30 min. at 37° with a 1/5 dilution of the appropriate sera (Ravid, Margalith & Goldblum, 1968; Goldblum, Ravid & Becker, 1968). The antisera were coupled with fluorescein isothiocyanate for 1 hr at room temperature. The γ-globulin fraction was separated on a DEAE cellulose column using phosphate buffer 0.01 M, pH 7.2, supplemented with 0.15 M-NaCl. Monkey antiserum to SV40 was kindly supplied by Dr J. L. Melnick.

Complement fixation. A modified Kolmer (Gradwohl, 1956) complement-fixation method was employed to test for the presence of T antigen. Immune sera for the com-
Transformation of BSC t cells

Complement-fixation test were obtained by bleeding hamsters bearing large tumours induced by HK-7 cells. Reference serum was obtained from Flow Laboratories Ltd, Scotland. Crude tumour antigen was prepared from HK-7 and BSC/SV40 cells according to the method described previously (Fuks et al. 1967).

RESULTS

Establishment of the BSC/SV40 carrier state

Fully grown BSC t cell monolayers in tubes were washed and infected with various multiplicities of SV40 ranging from 100 to 0.1, and incubated at 37°. During the 3 weeks following infection, the great majority of the cells exhibited a typical cytopathic effect (vacuolization) and the cells detached from the glass. A small number of cells, however, remained unaffected and slowly developed into colonies. Colonies appeared in cell cultures infected with multiplicities of 100 and 10, whereas at lower multiplicities none were visible. The colonies developed rapidly into cell monolayers and these were passaged and tested for infectious virus, and virus and tumour antigens.

The cells in the colonies multiplied and formed monolayers although the medium contained between 10^6 and 10^7 p.f.u. of SV40 virus. A number of such monolayers were passaged several times, found positive for infectious virus, and virus and tumour antigens, and designated BSC/SV40.

<table>
<thead>
<tr>
<th>Table 1. Infectious virus and virus and tumour antigens during passage of BSC/SV40 cell cultures</th>
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<tbody>
<tr>
<td>Passage no.</td>
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<tr>
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</tr>
<tr>
<td>7</td>
</tr>
<tr>
<td>12</td>
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<td>15</td>
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<tr>
<td>17</td>
</tr>
<tr>
<td>21</td>
</tr>
<tr>
<td>36</td>
</tr>
<tr>
<td>44</td>
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Infectious virus and virus and T antigens during passage of the BSC/SV40 carrier cultures

The number of cells producing infectious virus was determined by the infectious centre assay. This was done by plating various numbers of BSC/SV40 cells on BSC t monolayers in 60 mm. Falcon Petri dishes. Before plating, the BSC/SV40 monolayers were washed three times with Hanks’s solution, treated with EDTA, incubated for 1 hr with antiserum to SV40, washed again three times, counted in a haemocytometer and plated on BSC t monolayers. After 1 hr of adsorption at 37°, the monolayers were overlaid as described in Methods. The percentage of cells containing virus antigen was determined by the immunofluorescent technique. The tumour antigen was tested by the immunofluorescent and complement-fixation methods. Two to three thousand cells were examined by the immunofluorescent technique to estimate the percentage of cells containing tumour antigen. The samples tested by the complement-fixation method contained 10^7 cells/ml.
At the 7th and 12th passage levels every cell in the BSC/SV40 carrier culture was able to produce infectious virus (Table 1). The proportion of cells producing infectious virus decreased continuously on passage. Only 2% of cells produced virus at passage 21 and 0.2% at passage 44. The proportion of cells exhibiting virus antigen was 4.2 at passage no. 12, which dropped to 0.025 at the 36th passage and to 0.01% at the 44th passage. The proportion of cells exhibiting tumour antigen rose from 4% at the 7th passage, increasing during the passages of the BSC/SV40 cultures to 100% at passage 44. The high proportion of cells containing tumour antigen (99.5 to 100%) was retained following subsequent passages. The complement-fixation titre of the tumour antigen at the 4th passage was low but increased, reaching to 32 to 64 at passage level 36, parallel with the increase in the proportion of cells exhibiting tumour antigen by the immunofluorescent technique. Thus, during passage of the BSC/SV40 virus carrier cultures a ‘transformed state’ was established.

In the immunofluorescence test, the BSC transformed cells exhibited a marked difference from hamster kidney transformed cells (HK-7) as well as from BSC1-infected cells in the morphological appearance of the tumour antigen. In the HK-7 transformed and BSC1-infected cells, the tumour antigen stained homogeneously whereas in the BSC transformed cells the fluorescent staining appeared in a distinct granular form (Plate 1).

Table 2. Effect of SV40 antiserum, actinomycin D and cytosine arabinoside on virus (V) and tumour (T) antigens in BSC/SV40 and BSC1 transformed cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Carrier cells</th>
<th>Transformed cells</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>T</td>
<td>V</td>
</tr>
<tr>
<td>Control</td>
<td>4.52</td>
<td>3.95</td>
</tr>
<tr>
<td>SV40 antiserum</td>
<td>0.00</td>
<td>0</td>
</tr>
<tr>
<td>Actinomycin D</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Cytosine arabinoside</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* In a sample taken at 5 days 0.01% of the cells fluoresced.

Effect of antiserum to SV40, actinomycin D and cytosine arabinoside

These reagents were tested on both the carrier and the transformed cells. For testing the effect of antiserum, the cells were grown and passaged three times in the presence of 1/100 dilution of antiserum to SV40 and the proportion of cells containing virus and tumour antigens determined by immunofluorescence. The effect of actinomycin D and cytosine arabinoside was tested on BSC1 monolayers in Leighton tubes, in which the medium was changed, inhibitors added and samples withdrawn 3 to 5 days after addition of the inhibitors. The samples were tested for the presence of tumour and virus antigens. In the carrier cells, antiserum to the virus, actinomycin D and cytosine arabinoside completely inhibited the synthesis of both virus and tumour antigens, whereas in the transformed cells they affected the virus antigen only and the tumour antigen remained unaffected (Table 2). It seems that in the virus carrier cells, synthesis of T antigen is linked to viral multiplication and therefore both are inhibited by antiviral SV40 serum, CA and AD. This is not so in the transformed cells.
Immunofluorescence of tumour (T) antigen in BSC 1 cells infected with SV 40 (a), and in BSC 1 transformed cells (b).
Superinfection of the BSC I transformed cells

The susceptibility of BSC I transformed cells to superinfection with heterologous viruses (herpes simplex, attenuated poliovirus type 1 and vaccinia) and with the homologous SV40 virus was tested (Table 3). The viruses were titrated in parallel on BSC I transformed and BSC I control cell monolayers in Petri dishes. The heterologous viruses exhibited the same titre on BSC I transformed and BSC I control monolayers. On the other hand the BSC I transformed cells were completely resistant to $10^6$ p.f.u. of the homologous SV40 virus.

Table 3. Superinfection of BSC I transformed cells

<table>
<thead>
<tr>
<th>Virus</th>
<th>BSC I transformed cells</th>
<th>BSC I control cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>SV40</td>
<td>0.0</td>
<td>6.4</td>
</tr>
<tr>
<td>Herpes simplex</td>
<td>6.9</td>
<td>6.9</td>
</tr>
<tr>
<td>Poliovirus type 1</td>
<td>6.8</td>
<td>6.9</td>
</tr>
<tr>
<td>Vaccinia</td>
<td>5.5</td>
<td>5.5</td>
</tr>
</tbody>
</table>

Selection of virus-free clones

Virus-free clones were selected from BSC I transformed cell cultures at the 45th passage. At this passage level all the cells contained tumour antigen and $0.005\%$ virus antigen. The monolayers were treated with EDTA and a small number of cells seeded in 60 mm. Falcon Petri dishes in Eagle’s modified medium supplemented with 10% calf serum. Ten days later, 15 discrete cell colonies were removed, and single cells of each grown into monolayers. They were tested for virus and tumour antigens by the immunofluorescent technique. Thirteen of the colonies had tumour antigen in all the cells but no detectable virus antigen. The thirteen colonies were further passaged and the fourth passage material tested for virus antigen. Only two clones were free of detectable virus antigen and exhibited T antigen in all the cell nuclei. These two virus-free cell clones were further passaged and cells and supernatant fluids of the 8th passage tested for infectious virus. Whole and sonic-treated cells at concentrations of $10^8$ and $10^7$ ml. were plated on BSC I monolayers. No virus could be detected in these clones.

The‘efficiency of cloning’ of the transformed virus-free clones was tested in parallel with cells of the virus carrier state (BSC/SV40). The cells were plated in Eagle’s modified medium supplemented with 10% calf serum. Two weeks after cloning the colonies were fixed, stained with Giesma and counted. The virus-free cloned cell line had a 90% efficiency of cloning and its colonies were large, whereas the virus carrier cells had only 7% efficiency of cloning and formed small colonies.

DISCUSSION

Cells infected with SV40 can either undergo cytolysis as a result of virus multiplication or become transformed. In SV40 infection, these two phenomena do not usually occur in the same type of cells. Unlike SV40, polyoma virus may cause both cytolysis
and transformation in the same cell system (Medina & Sachs, 1960; Vogt & Dulbecco, 1960). This report describes the transformation of monkey kidney cells (the BSC1 cell line) which usually undergo cytolsis when infected with SV40. The transformation followed a chronic infection of BSC1 cells with the virus, the BSC/SV40 virus carrier state.

The BSC/SV40 virus carrier state could be established only by infecting BSC1 monolayers with a high multiplicity of infection. The virus carrier state could be obtained repeatedly and at will when a high m.o.i. was used. It is possible that interference played an important role in the establishment of the system. Auto-interference phenomena have been reported to occur frequently in SV40 infections (Black, Crawford & Crawford, 1964; Uchida, Watanabe & Kato, 1966; Uchida et al. 1968).

The state of the virus genome in the SV40 transformed BSC1 cells is at present under investigation. In preliminary experiments, fusion with susceptible cells did not cause the activation of SV40 virus (M. Margalith et al. unpublished results; Dr F. Rapp, personal communication). The integration of the virus genome in these transformed cells may, therefore, be of a different nature from that in SV40 transformed cells in which cell hybridization causes reactivation of the virus (Watkins & Dulbecco, 1967; Westphal & Dulbecco, 1968; Stephalski, Knowles & Koprowski, 1968).

REFERENCES


Transformation of BSC I cells


(Received 17 March 1969)