Abortive Infection and Transformation of Human Embryonic Fibroblasts by *Molluscum contagiosum* Virus

By G. BARBANTI-BRODANO, A. MANNINI-PALENZONA, O. VAROLI, M. PORTOLANI AND M. LA PLACA

*Istituto di Microbiologia, Università di Bologna, Bologna, Italy*

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**SUMMARY**

Abortive transformation of human embryonic fibroblasts was induced by *Molluscum contagiosum* virus. The parameters studied in order to test the establishment of a persistent infection and transformation of cells were: (1) virus interference and interferon production; (2) alterations of cell morphology and growth; (3) presence of neoantigens; (4) karyotype. The possible mechanisms leading to an abortive transformation are discussed.

**INTRODUCTION**

*Molluscum contagiosum* virus (MCV) belongs to the poxvirus group and is the causative agent of small tumours arising in the skin of man (Postlethwaite, 1970). Although MCV has not yet been isolated and propagated in tissue culture, it can infect cells which undergo typical cytopathic changes without production of progeny infectious virus (Dourmashkin & Febvre, 1958; Neva, 1962). The analysis of the steps leading to this abortive infection has shown that virus attaches to cells, penetrates and is uncoated up to the core stage (Postlethwaite, 1970). Evidence has been provided that the c.p.e. produced by MCV in cell cultures is dependent upon the integrity of the virus genome and upon protein synthesis (La Placa, 1966; La Placa et al. 1967a), which suggests that the expression of some virus functions is necessary to induce c.p.e.

Because MCV is naturally oncogenic *in vivo*, as it leads to hyperplastic proliferation of the epidermis and to the appearance of tumours, and because it produces *in vitro* an abortive infection supported by the partial expression of the virus genome, it seemed to be interesting to determine whether this virus is capable of inducing cell transformation *in vitro*.

We have tested this possibility by studying MCV-infected cells with regard to several properties generally accepted as typical markers of *in vitro* transformed cells. The cells chosen for these experiments were human embryonic fibroblasts, which have a limited life span and do not undergo spontaneous transformation (Hayflick, 1965).

**METHODS**

*Cells.* Human embryonic fibroblasts (HEF) were obtained by trypsinization of an 8-week-old embryo. Cells were grown in Eagle's minimum essential medium (MEM) supplemented with 10% foetal bovine serum (FBS); they were maintained in passages at a splitting ratio of 1:2 and were used between the 8th and 14th passages. Primary human amnion (HA) cells were obtained by trypsinization of the amniotic membrane and grown in MEM plus 10% calf serum (CS). KB cells were grown in Hanks' medium plus 10% CS.
Viruses. A pool of MCV was prepared by homogenization of nodules obtained from human patients. The tissue was suspended in Hanks' solution at 10% (w/v), then was ground in a mortar with quartz sand and the homogenate was centrifuged at 800 g for 15 min. The supernatant fluid was found to contain typical brick-shaped virus particles when observed by negative staining under an electron microscope; it represented the virus inoculum and had a titre of 10^8 TCID_{50} per ml in HA cells. Vesicular stomatitis virus (VSV) was grown in HEF (titre 10^6.8 p.f.u./ml) and vaccinia virus, IHD strain, was grown in KB cells (titre 10^7.1 p.f.u./ml).

Infection of cells with MCV. HEF grown at confluence were infected with MCV at different input multiplicities. Virus was adsorbed at 37 °C for 2 h; then the cultures were fed with MEM plus 2% FBS. The morphological alterations which appeared in the cell monolayer after infection were observed directly in the culture or after fixation in methanol and staining by the May–Grünewald–Giemsa method. The mitotic index was determined by counting cells in mitosis over 100 microscopical fields, corresponding to 30,000 cells.

Production of immune serum. MCV-infected HEF (5 x 10^6) were harvested with 0.1% EDTA in phosphate-buffered saline solution (PBS) without Ca++ and Mg++ 24 to 48 h after infection, when showing maximum c.p.e. Cells were washed three times and suspended in 1 ml of PBS. The cell suspension was thoroughly mixed with an equal amount of complete Freund adjuvant and the emulsion was injected in the foot pads of a guinea pig. Inoculations were repeated twice both in the foot pads and subcutaneously in the back of the animal at a week interval, and the animal was bled one week after the last injection. To remove antibodies directed to antigens of normal HEF from the immune serum, 1 ml of serum was adsorbed with 3 x 10^7 normal HEF twice at room temperature for 30 min and once at 37 °C for 1 h.

Immunofluorescence tests. Immunofluorescence tests on viable cells were performed according to Möller (1961) and Palm, Heyner & Brinster (1971). 5 x 10^6 MCV-infected or normal HEF were detached from the glass with 0.1% EDTA in phosphate-buffered saline solution (PBS) without Ca++ and Mg++ and washed three times in PBS. The cell pellet was then resuspended in 0.2 ml of immune serum at various dilutions and incubated at 37 °C for 30 min. Cells were then washed three times in PBS and resuspended in 0.2 ml of fluorescein-conjugated rabbit antiserum to guinea pig immunoglobulins (Hyland, Los Angeles, California) diluted 1:5 in PBS. After 30 min incubation at 37 °C, cells were washed three times in PBS, suspended in two drops of buffered glycerol and observed for immunofluorescence in a u.v. microscope. Immunofluorescence tests were carried out also on acetone-fixed cells.

Test for virus interference and interferon production. MCV-infected and normal HEF were grown at confluence in Petri dishes and infected with 50 to 100 p.f.u. of VSV or vaccinia virus per Petri dish. After 1 h adsorption at 37 °C, the inoculum was aspirated and the cultures were washed three times with MEM. Cells infected with VSV were overlaid with MEM containing 2% FBS and 1% agar and cells infected with vaccinia virus were overlaid with the same medium containing 1% methylcellulose (Koch–Light, Colnbrook, England). After 48 h incubation at 37 °C, cells were stained with neutral red or crystal violet and plaques were counted.

To test interferon production by MCV-infected cells, the supernatant fluids from cultures at different times after infection and the supernatant fluids from normal cultures were brought to pH 2] with 0.05 M-HCl in order to inactivate the residual unadsorbed virus (Postlethwaite, 1964) and to assess the resistance of the soluble interfering material to acid pH. After overnight incubation at 4 °C, the pH was adjusted to 7.2 with 0.1 M-NaOH. The materials were then centrifuged at 100,000 g for 4 h. The supernatant fluid was decanted.
and its antiviral spectrum and cell specificity were tested. It was shown to reduce the plaque count of VSV, herpes simplex and parainfluenza type 3 viruses in HEF. It did not reduce the number of plaques formed by VSV in BHK-21 cells. Monolayer cultures of normal HEF were incubated at 37 °C for 18 h with 2 ml of different dilutions of treated supernatant fluids. Cells were then washed three times and infected with VSV.

**Growth of cells in a semi-solid medium.** The technique was that described by Stoker (1968). MCV-infected and normal HEF were suspended at a concentration of 1.5 x 10^6 cells/ml in MEM plus 10 % FBS and 1 % methylcellulose. The cell suspension was seeded on 50 mm Petri dishes lined with MEM containing 10 % FBS and 1 % agar. The cultures incubated at 37 °C were observed daily for the presence of colonies. Colonies and single cells were counted 6 days after seeding.

**Karyology.** Chromosomes were examined by the technique of Moorhead & Nowell (1964).

**RESULTS**

**Morphological alterations of MCV-infected cells**

Adsorption and penetration of MCV to infected HEF was monitored by electron microscopy. Two hours after infection many virus particles were adsorbed to the cell surface and engulfed into cells by phagocytosis; a few particles were already undergoing first stage uncoating into the cytoplasm of infected cells (Fig. 1(a)). Eight hours after infection most of the intracytoplasmic virus particles were in the core stage (Fig. 1(b)).

HEF infected by MCV developed a typical c.p.e., characterized by rounding, swelling and clumping of cells, within 24 to 48 h after infection (Fig. 2(c), (d)). These lesions persisted for 2 to 3 days without cell lysis, then regressed leaving a morphological transformation of cells that changed to a polygonal shape and grew in multilayered 'foci' with apparent loss of contact inhibition. The latter modifications lasted about one month in resting cultures and were propagated through three passages when cells were split (Fig. 2(e), (f)). After that period the 'foci' of epithelial-like cells disappeared and cells resumed a fibroblastic morphology.

**Stimulation of cell division and restoration of contact inhibition during propagation of MCV-infected cells**

Normal HEF and HEF at the fourth passage after infection with MCV were seeded on coverslips and stained by the May–Grünwald–Giemsa method at 24, 48 and 72 h after seeding. At 24 and 48 h after seeding, when cells grew sparsely without reaching confluence, the count of cells in mitosis showed that dividing cells in the culture of MCV-infected HEF were more than twice as many as in normal controls; at 72 h after seeding, when cells started to reach confluence, the number of mitoses in the culture of MCV-infected cells dropped to the level of the normal controls (Fig. 3).

**Growth of cells in a medium containing methylcellulose**

Cultivation of cells in a medium containing methylcellulose showed that 48 % of cells at the third passage after MCV infection and 14 % of control cells had grown into colonies. Moreover, the size of colonies produced by the infected cells was remarkably larger than that of colonies produced by normal cells (Fig. 4). Upon trypsinization cells were counted in each of 20 colonies from normal and infected cultures. It was found that on the average 44.8 cells made up colonies in MCV-infected cultures and 64 cells made up colonies in normal cultures. The number of single cells plus colonies was 1.33 x 10^9/ml in the culture of MCV-infected cells and 1.29 x 10^9/ml in the culture of normal cells.
Fig. 1. Early phases of infection of HEF by MCV. (a) Virus particles adsorbed to the cell surface and in the process of being phagocytosed (2 h after infection); (b) intracytoplasmic cores in different stages of uncoating (8 h after infection).

Cells and colonies of both MCV-infected and normal cells when taken from the culture in methylcellulose and seeded in Petri dishes gave rise to monolayers of cells with a typical fibroblastic morphology.

**Virus interference**

Tests for virus interference showed that the number and the size of plaques produced by VSV on cells at different passages after infection with MCV were reduced by 50% or more
MCV infection and transformation of HEF

Fig. 2. Morphological appearance of MCV-infected HEF. (a), (b) normal HEF. (c), (d) HEF 48 h after MCV infection. (e), (f) HEF at the third passage after MCV infection. Left side: unstained. Right side: stained by the method of May-Grünwald-Giemsa. Magnification $\times 75$.

as compared to the normal controls. On the contrary, the number of plaques produced by vaccinia virus was increased in MCV-infected cells. Differences in plaque production between MCV-infected cells and normal controls were apparent up to the sixth passage after infection with MCV (Table 1).
Fig. 3. Number of dividing cells in cultures of normal HEF and MCV-infected HEF. 
- - , HEF at the fourth passage after MCV infection; O - O, normal HEF.

Fig. 4. Growth into colonies of (a) HEF at the third passage after MCV infection and 
(b) normal HEF. Magnification ×45.
Table 1. **Superinfection of MCV-infected cells with VSV or vaccinia virus**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Cells</th>
<th>No. of plaques per dish*</th>
<th>VSV†</th>
<th>Vaccinia virus</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>MCV-infected cells, 1st passage</td>
<td>60.2 ± 5.4</td>
<td>41.2 ± 2.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Normal cells</td>
<td>Confluent</td>
<td>32.6 ± 1.8</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>MCV-infected cells, 3rd passage</td>
<td>27.6 ± 2.2</td>
<td>58.4 ± 3.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Normal cells</td>
<td>48.4 ± 3.2</td>
<td>37.2 ± 2.8</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>MCV-infected cells, 4th passage</td>
<td>39.1 ± 2.7</td>
<td>39.5 ± 3.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MCV-infected cells, 5th passage</td>
<td>28.2 ± 4.4</td>
<td>38.1 ± 4.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MCV-infected cells, 6th passage</td>
<td>55.5 ± 6.0</td>
<td>19.3 ± 1.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Normal cells</td>
<td>90.3 ± 7.6</td>
<td>24.7 ± 1.9</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>MCV-infected cells, 6th passage</td>
<td>50.7 ± 4.3</td>
<td>51.8 ± 4.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Normal cells</td>
<td>65.0 ± 3.8</td>
<td>51.4 ± 3.7</td>
<td></td>
</tr>
</tbody>
</table>

* Average of the number of plaques counted in 2 to 4 Petri dishes.
† Plaques produced by VSV on MCV-infected cells were about half the size of plaques produced on normal cells.

Table 2. **Interferon production by MCV-infected cells**

<table>
<thead>
<tr>
<th>First experiment</th>
<th>No. of plaques</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEF + MCV supernatant fluid (2nd passage). Undiluted</td>
<td>81.3 ± 7.6</td>
</tr>
<tr>
<td>HEF + MCV supernatant fluid (3rd passage). Undiluted</td>
<td>88.6 ± 2.4</td>
</tr>
<tr>
<td>HEF + normal supernatant fluid. Undiluted</td>
<td>118.5 ± 5.0</td>
</tr>
<tr>
<td>HEF</td>
<td>122.1 ± 9.2</td>
</tr>
</tbody>
</table>

| Second experiment | |
|-------------------||
| HEF + MCV supernatant fluid (5th passage) 1:4 | 37.5 ± 0.5 |
| 1:8               | 47.5 ± 2.5 |
| 1:16              | 57.5 ± 0.5 |
| 1:32              | 59.5 ± 3.1 |
| HEF + normal supernatant fluid 1:4 | 61.3 ± 2.8 |
| HEF | 63.5 ± 3.5 |

Interferon production assays showed that the number of plaques produced by VSV in normal HEF was reduced when cells were pre-treated with supernatant fluids of MCV-infected cells at various passages after infection (Table 2).

**Detection of neoantigens on MCV-infected cells by immunofluorescence**

Immune guinea pig serum extensively absorbed with normal HEF produced a patchy membrane fluorescence on 80% of the cells at the third passage after MCV infection, when used undiluted or diluted 1:2 (Fig. 5). A weak fluorescence was observed at the same dilutions on 10% of cells in the normal controls. No fluorescence was observed at higher serum dilutions on MCV-infected or normal HEF and when MCV-infected and normal HEF were stained with immune serum after fixation in acetone. No fluorescence was produced on MCV-infected cells by guinea pig pre-immune serum. Positive fluorescence produced by immune guinea pig serum could be blocked by rabbit hyperimmune serum to guinea pig immunoglobulins added before the fluorescein-conjugated serum.

**Results of cytogenetic studies**

The analysis of the karyotype of HEF at the third and fifth passages after MCV infection showed that the infected cells were euploid and the characteristics of chromosomes were indistinguishable from those of the controls.
Long term survival of MCV-infected cells

MCV-infected and normal HEF were passaged once a week at the splitting ratio of 1:2 in order to explore the possibility of establishing a continuous culture of transformed cells. Both MCV-infected and normal cells went into crisis at the 23rd passage and died out at the 25th passage, even though normal cells disappeared from the culture very soon, whereas MCV-infected cells survived for three months at the 25th passage level forming small colonies and eventually died.

DISCUSSION

The abortive infection of HEF by MCV leads to the persistent expression of some virus-specific functions in infected cells. This is indicated by the capacity to give interference and to produce interferon showed by infected cells at several passage levels after MCV infection. These results are consistent with the observation of Friedman-Kien & Vilěk (1967), that maximum production of interference and interferon is detected late (about 5 days after infection) in primary infection of cells with MCV. The enhancement of virus yield upon superinfection of MCV-infected cells by vaccinia virus suggests that complementation arises between defective virus particles present in the vaccinia virus inoculum and MCV or the MCV genome resident in MCV-infected cells. However, it should be taken into account that, in spite of the well documented efficiency of complementation among members of the poxvirus group (Fenner & Woodroofe, 1960; Joklik et al. 1960), this hypothesis is weakened by the failure of MCV to induce thymidine kinase synthesis (La Placa et al. 1967a) and to reactivate other poxviruses (La Placa, Portolani & Rosa, 1967b).

On the other hand, differences in plaque counts by VSV and vaccinia virus in MCV-infected and normal cells cannot be attributed to differences in virus adsorption because titration of the virus inoculum from MCV-infected and normal cells after a 2 h adsorption period gave the same plaque count.

Our results contrast with the data by Pirie et al. (1971) who found inhibition of vaccinia virus growth upon superinfection of MCV-infected mouse embryo fibroblasts. This discrepancy may be due to the different time interval elapsed before superinfection or to the different cell systems used. In other experiments we found that co-infection of HEF with MCV and vaccinia virus led to an enhancement of vaccinia virus yield.
As to surface neoantigens detected on MCV-infected cells, they should differ from structural virus antigens observed in the cytoplasmic membrane of cells infected by other poxviruses (Ueda, Ito & Tagaya, 1969) because the virus does not express late functions. It cannot be decided at present whether they are early virus coded products or components of the normal cytoplasmic membrane exposed by a virus-specific uncovering mechanism (Häyry & Defendi, 1970).

Moreover, HEF infected by MCV show distinct alterations in cell morphology and growth similar to those appearing in cells transformed by papovaviruses and oncogenic riboviruses. Indeed, MCV-infected cells exhibit some of the features generally accepted for the definition of transformed cells (Macpherson, 1970): (1) change of cell morphology from a fibroblastic to an epithelial-like shape; (2) stimulation of cell division; (3) growth in ‘foci’ and aggregates with loss of contact inhibition; (4) capacity to grow and form colonies in a semisolid medium; (5) presence of neoantigens.

The euploidy of MCV-infected cells still fits the transformed state of cells since no alterations of the karyotype were observed in several virus-transformed cell lines in the first passages after the establishment of transformation, or even during all their life span (Macpherson, 1970).

Such transformation, however, appears to be transitory since the normal fibroblastic morphology is regained by the cells at about the fourth passage after infection. At the same time contact inhibition is also restored and the stimulation of cell division ceases. Finally, cells infected by MCV do not show a longer survival in comparison with uninfected controls and do not give rise to a continuous cell line.

These characteristics are reminiscent of the abortive transformation commonly induced by papovaviruses and thoroughly described by Stoker (1968) in BHK cells infected by polyoma virus. In abortive transformation the transformed cell phenotype is mediated by physiological changes induced by a virus genome which is not integrated in the cell genome. Under such conditions the free virus genome is probably incapable of autonomous replication in the non-permissive cell and is eventually lost, allowing the cell to revert to the normal phenotype.

In the cell-virus system used in these experiments the appearance of abortive transformation might be due to several alternative conditions preventing the integration of the virus genome into the cell genome: (1) virus infectious cycle restricted to the cytoplasm; (2) lack of uncoating beyond the core stage; (3) exceedingly large size of the virus DNA (mol. wt. 180 to 200 × 10^6). In this connection it is noteworthy the observation of Roosa & Bailey (1970) that the transforming efficiency of cellular DNA on mammalian cells is increased after fragmentation by ultrasonic treatment.

This report confirms the possibility of achieving transformation of cells with members of poxvirus group, in agreement with the data reported by Koziorowska, Wlodarski & Mazurowa (1971) who established a carrier state of vaccinia virus in mouse embryo fibroblasts followed by neoplastic transformation.

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REFERENCES


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