Transformation of Bovine Cell Cultures by Preparations of Polyoma Virus

(Accepted 25 September 1966)

Transformation, i.e. heritable morphological and growth changes, of cultured bovine cells induced by bovine papilloma virus has been reported by several authors (1, 2). We report here the finding that suspensions containing polyoma virus which has the ability to cause a transformation in cultured cells of several species of rodents (3–6) can also cause proliferation and various persistent alterations in certain bovine tissue culture cells.

The cellular changes were first recognized in two clones of diploid cells, P-3 and P-6, obtained from cultures of foetal bovine skin (7), after exposure to two different preparations of plaque-purified polyoma virus (Toronto small-plaque strain). The cells were seeded in 50 mm. diameter plastic Petri dishes (2 x 10^6 cells per dish) and were grown and maintained at 36° in a humidified atmosphere of 5 % (v/v) CO₂ in air. The medium consisted of modified Eagle’s medium containing additional amino acids and vitamins (8) supplemented with 10 % (v/v) unheated foetal calf serum, penicillin (100 u./ml.) and streptomycin (100 µg./ml.). Freshly seeded cultures of uninfected clones P-3 and P-6 consisted of polygonal and triangular cells of epithelioid appearance. After 24 hr, 2 ml. of a suspension of polyoma virus, grown in mouse-embryo cultures and prepared as described by Crawford (9), were added to the Petri dishes. The titre of the viral suspension was 2 x 10⁸ p.f.u./ml. The virus was left in contact with the cells for 18 hr at 36°. The inoculum was then removed, the cell sheet washed three times with medium and 4 ml. of fresh medium added to each culture. Control cultures of clones P-3 and P-6 were inoculated with various uninfected mouse-embryo culture extracts prepared by the same method. Fluids were changed every 4 days.

No degenerative change could be detected in control or inoculated cultures within a period of 60 days. After 24 to 48 hr, there was a marked increase in the number of cells in mitosis in the inoculated cultures, up to 5 times that seen in the controls; this difference persisted for 1 or 2 days, then declined over the next 2 or 3. From the 6th to 8th day, an increasing number of multinucleated cells, 3 to 4 times the size of normal cells, were seen in the inoculated cultures, and by the 12th to 14th day several areas of proliferative cells were observed. The new growth consisted of both epithelioid and fibroblastic cells growing actively in a disorganized pattern. By the 3rd to 4th week after inoculation, there were between 10 and 20 foci per culture, consisting of several layers of these active cells, together with large cells with one or more nuclei, and many round cells in small groups or in prominent clusters (Plate 1, fig. 1). With time, these foci increased somewhat in number and rather slowly in area: by 60 days there were up to 25 per culture. In some inoculated cultures such foci were only observed after a delay of 55 days. At later stages, spindle-shaped fibroblast-like cells with round cells on top of them tended to replace the original mixture of epithelioid and fibroblastic transformed cells in the foci.
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Over a period of 60 days, control cultures showed only slow and moderate acidification of the medium and no obvious sign of continuing cell multiplication. The cell sheet was quite uniform, consisting of a monolayer of triangular and polygonal epithelioid cells, disposed in a regular pattern.

One of the foci arising on an inoculated monolayer of the clone P-6 was isolated with a ring and trypsinized at the 24th day and the cells subcultured. A serial passage line (P-6/Py) was established with ease and has been subcultured 20 times at the time of writing. The predominant cell type is spindle-shaped, but epithelioid cells are also seen, and some multinucleated cells with macronuclei. There is a marked acidification of the medium and the cells multiply rapidly to form a continuous monolayer. Subsequently, they grow on top of one another, forming a multilayered sheet with clusters of round cells. During this final stage, many cells become detached from the sheet and float in the medium; they grow actively when seeded into a fresh dish. Transformed cultures are readily dispersed by 0.1 % (w/v) trypsin to give single cells. Control cells, when cultured in the same manner as the transformed cells, grow more slowly, cause less acidification of the medium, and show only an occasional mitotic figure and no piling up once a confluent monolayer has been achieved. The mean generation times of cell lines P-6 and P-6/Py during the logarithmic phase of growth are respectively 24 to 26 hr and 20 to 22 hr; and the saturation densities, after 15 days' growth with medium changes every 3 days, respectively 0.5 and 2.5 × 10⁵ cells/cm².

Coverslip cultures of the normal and transformed cells were stained with Giemsa's stain (Plate 1, figs. 2, 3). No intranuclear or cytoplasmic inclusion bodies were seen in transformed cells, but some of them had large nuclei with prominent nucleoli. Cytogenetic studies of cells from the 5th subculture of the transformed line P-6/Py showed a modal chromosome number of 60 as in the control cells. About 1 % of the mitotic cells examined were tetraploid.

P-6 and P-6/Py cells, seeded in 50 mm. diam. plastic Petri dishes at 100 cells per dish, gave a plating efficiency of 7.5 % for the transformed cells compared with 0.01 % for the control cells. When seeded in similar densities on X-irradiated mouse-embryo cells (8 × 10⁴/dish), P-6 and P-6/Py cells had plating efficiencies, respectively, of 15 to 30 % and 50 to 70 %. The appearance of individual colonies corresponded with the behaviour of the two cell lines in ordinary culture: colonies of P-6 consisted of a rather open network of monolayered cells, while those of P-6/Py were dense and multilayered.

Different amounts of polyoma virus were added, in the same conditions as above, to one of the foetal bovine skin cell clones, P-3. Foci of transformed cells were observed, varying in number with the virus dose, after intervals of from 12 to 60 days, in those cultures which had received not less than 10⁷ p.f.u. of polyoma virus, i.e. about 50 p.f.u. per cell (Fig. 1).

No haemagglutinin (HA) for guinea-pig red cells was detected in the supernatant of the control P-3 dishes. In the 4 ml. of medium from cultures inoculated with 2 × 10⁸ p.f.u. of polyoma virus in 0.2 ml. (HA titre of 1/80,000), a residual HA activity up to a dilution of 1/40 could be observed 4 days after exposure of the cells to virus. This activity thereafter disappeared.

Trypsin-dispersed P-6/Py cells of the 4th and 11th subcultures were seeded on to subconfluent secondary cultures of mouse-embryo cells in numbers varying from
10^8 to 10^9 cells per culture. Fluids and cells were harvested after 14 days and inoculated into further mouse-embryo cultures. No cytopathic effect was observed; nor was any polyoma virus infectivity or HA activity detected.

Virus neutralization tests were made with 4 x 10^8 p.f.u. of polyoma virus mixed with either a normal rabbit serum or antiserum from a rabbit which had been immunized with a preparation of uncloned large-plaque polyoma virus, purified by 3 extractions with Arcton. This antiserum neutralized 8 HA units of small-plaque polyoma virus at a dilution of 1/60,000. The virus + serum mixtures were held for 1 hr at 36°, as were also controls with sera alone, virus alone, or mouse embryo extract. They were then added to monolayers of clone P-3. After 45 days, no foci of transformed cells had appeared in the cultures inoculated with the mixture of antiserum + virus, or with embryo extract or either serum alone, whereas all the cultures receiving normal rabbit serum + virus, or virus alone, had developed foci of transformed cells.

![Graph](image-url)

Fig. 1. Transformation of bovine skin cells exposed to polyoma virus. Response of bovine clone P-3 cells 60 days after exposure to varying concentration of a preparation of small-plaque polyoma virus. ○, Focal count in individual culture; □, mean focal count of four cultures.

Thus, the transformation of the bovine cell clones appeared to be related to polyoma virus, or to some other agent or factor present both in the inocula used for cultures and in the material used for rabbit immunization. To characterize more precisely this transforming agent, a special stock of Toronto small-plaque polyoma virus was prepared. It was grown from a single plaque picked from a monolayer culture with 2 plaques, part of a titration using 10-fold virus dilutions. Control material was prepared from the same batch of mouse embryo cells inoculated with 10 samples of cells + agar picked from a culture of the next higher dilution in the series. The virus preparation produced typical transformed foci in cultures of two postnatal bovine skin cell clones, GC-1 and M-3 (7); whereas the control preparation produced no such foci. The focus forming capacity of the virus preparation was not impaired by heating at 60° for 30 min.: 10^8 p.f.u. of heated virus inoculated on to subconfluent cultures of 4 x 10^6 cells gave rise to 5 to 10 foci after 3 to 4 weeks. Treatment of the virus preparation with 20% (v/v) ether for 6 hr at 4° caused little or no loss of focus forming activity.
The transforming effect is probably therefore due to an agent in polyoma plaques which is relatively resistant to ether and to heat and which is probably polyoma virus itself (cf. 10). It should be noted that Diderholm & Wesslén (11) detected infectious polyoma virus in cultures of bovine cells exposed to polyoma DNA, but not in those exposed to whole virus. No transformation of bovine cells was observed with either type of inoculum (11, 12); but the doses of polyoma virus were lower than those used in the present experiments.

In preliminary tests using the soft-agar technique (13, 14) 3 to 7 colonies of about 50 or more cells appeared after 3 weeks in Petri dishes seeded with 10⁵ cells which had been exposed to 10⁸ p.f.u. of virus just before suspension in the agar; no such colonies were observed in unexposed controls. The formation of colonies in agar was prevented when virus was incubated with polyoma antiserum before being added to the cells. When colonies were picked from the agar and grown in liquid medium, they produced an outgrowth of cells which resembled in behaviour and morphology the transformed cells already described.

The inherited alteration in cell form, and in the rate and pattern of growth, suggests that the changes produced in these cloned bovine cells by the polyoma virus preparations are comparable to the transformation which they induce in hamster cells in vitro. Whether these altered bovine cells have oncogenic potentialities is not yet known. Further studies are in progress concerning the presence of polyoma viral or tumour antigen in such transformed cells, as well as the quantitative aspects of the interaction of cell and virus.

We are grateful to Professor Michael Stoker and Dr Ian Macpherson for their criticism and advice, and to Miss Anne M. Baker for her valued assistance. One of us (M.T.) was supported by a grant from the Medical Research Council.

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(Received 31 August 1966)
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EXPLANATION OF PLATE

Fig. 1. Transformation of bovine cells by polyoma virus. Edge of a focus of transformed cells with normal cells on left, 4 weeks after exposure of monolayer of bovine skin-cell clone P-6 to a preparation of small-plaque polyoma virus. Note piling-up of cells in focus, with scattered groups of round cells and occasional large multinucleate cells. × 120.

Fig. 2. Normal bovine cells. Seven-day culture of normal bovine skin-cell clone P-6. Fixed with formol saline and stained with Giemsa. × 300.

Fig. 3. Polyoma-transformed bovine cells. Seven-day culture of transformed bovine skin-cell line P-6/Py, derived from a proliferative focus arising on monolayer of clone P-6 exposed to small-plaque polyoma virus. Fixed with formol saline and stained with Giemsa. × 300.