Persistent Infection and Transformation of Mouse Glial Cultures by K Virus, a Murine Papovavirus

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SUMMARY

Foetal mouse glial cultures were inoculated with murine K papovavirus and subjected to serial subcultivation. Two cell lines were developed. The first of these, KVBCG2A, remained positive for viral infectivity and K virus capsid (V) antigen for over 30 subcultivations. Productive infection was not abolished by serial subcultivation in the presence of antiviral antibody. The second cell line, KVBCG1B, became negative for infectious virus and K virus V antigen, could be cloned from single cells and produced tumours in mice. Sera from tumour-bearing animals produced nuclear fluorescence of KVBCG1B cells and K virus-infected mouse embryo cells but did not react with uninfected mouse embryo cells or with cells infected by polyoma virus. DNA hybridization studies confirmed the presence of K virus DNA in KVBCG1B cells and suggested integration of the viral genome into host chromosomal DNA. K virus produces both persistent infection and cell transformation in glial cultures derived from its natural host.

The interaction of papovaviruses with human glial cells in vitro has been studied with interest since the discovery that two members of the papovavirus group, JC virus and simian virus 40 (SV40), are associated with a fatal demyelinating infection of immunosuppressed human patients, progressive multifocal leukoencephalopathy (PML) (Padgett et al., 1971; Weiner et al., 1972). PML is characterized by lytic infection of oligodendrocytes, with secondary demyelination, and by non-productive infection of astrocytes, which develop morphological features suggestive of cell transformation (Walker, 1978; Dörries et al., 1979). Shein (1967) reported that inoculation of primary cultures of human foetal glial cells with SV40 results in lytic infection of small, dark-staining cells, which he termed spongioblasts, and in both lytic infection and transformation of astrocytes. Subsequent work by Oster-Granite et al. (1978) demonstrated that SV40 also replicates in two cell populations not identified by Shein, neuroblasts and mesenchymal cells. Inoculation of human foetal glial cultures with a second papovavirus, BK virus, produces an infection essentially identical to that seen with SV40 (Oster-Granite et al., 1978). Serial passage of BK virus-infected glial cultures results in the appearance of both persistently infected and transformed cell lines (Takemoto et al., 1979). JC virus, the agent most frequently associated with PML, was initially isolated in primary human foetal glial cultures and has been reported to replicate almost exclusively in spongioblasts or oligodendroblasts (Walker, 1978).

Although infection of glial cultures by SV40, BK and JC viruses has been described in considerable detail, little is known about the interaction of other papovaviruses with glial cultures derived from their natural hosts. We have previously reported that K virus, a murine papovavirus, is capable of limited replication in primary cultures of foetal mouse glial cells (Greenlee et al., 1982). The present study was conducted to determine whether foetal mouse glial cells inoculated with K virus develop persistent infection or undergo transformation.
Preparation of crude and gradient-purified K virus stocks and production of antisera to crude K virus and to K virus capsid (V) antigen were carried out as previously described (Greenlee, 1979, 1981). Polyoma virus for control experiments was obtained from the American Type Culture Collection, grown in mouse embryo cell cultures, and purified using the method of Crawford (1969). Rabbit antisera to polyoma virus V antigen was prepared by hyper-immunizing rabbits with u.v.-inactivated, gradient-purified virus (Greenlee, 1981). Antiserum to polyoma virus T antigen was a gift of Dr M. Israel, NIH. Haemagglutination (HA) and haemagglutination-inhibition (HI) determinations were conducted as previously described (Greenlee, 1979).

Pregnant inbred BALB/c mice were demonstrated to be without antibody to K virus or polyoma virus (Greenlee, 1979). Embryos were removed aseptically from these animals at 15 to 18 days gestational age. Spongioblast-rich cultures of foetal glial cells (Weiner et al., 1972) were grown in flasks and on coverslips in Eagle's MEM with 10% foetal calf serum, 200 units/ml penicillin and 200 μg/ml streptomycin at 37 °C in 5% CO₂. Cultures at 75% confluence were washed three times with Hanks' balanced salt solution, incubated for 2 h at 37 °C with 10⁵ haemagglutinating units of K virus, grown to confluence, trypsinized, and seeded into daughter flasks at ratios of 1:6. Daughter flasks were then serially subcultivated with twice weekly examination for evidence of c.p.e. or cell transformation. Coverslip cultures were prepared at each subcultivation for fluorescent antibody studies. Uninfected flasks of BALB/c glial cells similarly trypsinized and subcultivated served as controls.

Fluorescent antibody studies for K virus V antigen and for polyoma virus V and T antigens were conducted as previously described (Greenlee, 1979). Cryostat sections of lungs from animals which had died of K virus pneumonia and coverslip cultures of mouse embryo cells inoculated with K virus or polyoma virus served as positive controls. Uninfected coverslip cultures of foetal mouse glial cells and normal mouse or rabbit sera served as cell and serum controls. Media and cell extracts from inoculated flasks were assayed for K virus infectivity in mouse embryo cells (Greenlee et al., 1982). Infected glial cells were tested for their ability to survive multiple subcultivations, to grow in serum-free medium, to form colonies in soft agar, and to grow from single cells in microtitre plates (Greenlee et al., 1977). Total cellular DNA was extracted from transformed cells (Gross-Bellard et al., 1973) and digested with restriction endonucleases BamHI, Xhol, BglI or EcoRI which respectively cleaved the K virus genome at 0, 1, 2 or 3 sites (Law et al., 1979). The digested DNAs were subsequently electrophoresed in 0.6% agarose (Seakem) horizontal slab gels (Bethesda Research Laboratories) in Tris–acetate buffer (40 mM-Tris pH 7.8, 50 mM-sodium acetate, 1 mM-EDTA) at 2.5 V/cm for 16 h. After electrophoresis, DNAs in the gels were depurinated, denatured and transferred onto nitrocellulose membranes (Schleicher & Schüll, type HA85) (Wahl et al., 1979; Southern, 1975). K virus ³²P-labelled DNA probe (sp. act. 1 × 10⁸ c.p.m./μg) was prepared by labelling in vitro (Rigby et al., 1977) of DNA extracted from purified virions (Law et al., 1979). Hybridization was performed for 20 h at 60 °C with 3 × sodium saline citrate (0.45 M-NaCl, 0.045 M-sodium citrate), 10 × Denhardt's reagent, 100 μg/ml sonicated, denatured calf thymus DNA, 10% sodium dextran sulphate (Wahl et al., 1979; Law et al., 1981) and 5 × 10⁶ c.p.m. of ³²P-labelled denatured K virus DNA. After hybridization, the nitrocellulose blots were washed, air-dried, and exposed for 21 days at −70 °C to XAR X-ray films (Kodak) with intensifying screens.

Uninfected flasks of BALB/c glial cells could not be maintained beyond 10 to 12 subcultivations. Flasks inoculated with K virus initially showed occasional, small foci of c.p.e. consisting of rounding up of cells. Monolayers remained intact, however, and flasks ceased to exhibit c.p.e. within 2 or 3 subcultivations. Cultures grew slowly for 7 or 8 subcultivations, after which islands of small, relatively uniform cells appeared in one of the K virus-infected flasks (KVBCG2A). These cells rapidly became the predominant cell type and were subcultivated over 30 times without alteration in morphology or growth properties. Immunofluorescent staining of coverslip cultures of KVBCG2A cells revealed large numbers of cells exhibiting nuclear fluorescence specific for K virus V antigen up to the 25th subcultivation and somewhat smaller numbers of positive cells up to the 30th subcultivation (Fig. 1). High titres of K virus infectivity
Fig. 1. KVBCG2A cells stained at the 31st subcultivation with antisera to K virus V antigen, using indirect immunofluorescence methods. Bright nuclear fluorescence indicative of persistent K virus infection is present in three cells. Magnification × 300.

Fig. 2. KVBCG1B cells overlaid at the 33rd subcultivation with sera from mice developing tumours after inoculation with KVBCG1B cells, and stained using indirect immunofluorescence methods. The majority of cells exhibit nuclear fluorescence with nucleolar sparing. Magnification × 300.

Table 1. Virus content of cells and medium from KVBCG2A cells during serial subcultivation

<table>
<thead>
<tr>
<th>Passage no.</th>
<th>Cells</th>
<th>Medium</th>
</tr>
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<tbody>
<tr>
<td>10</td>
<td>$10^{-6.0}$</td>
<td>$10^{-4.8}$</td>
</tr>
<tr>
<td>26</td>
<td>$10^{-9.0}$</td>
<td>$10^{-5.8}$</td>
</tr>
<tr>
<td>31</td>
<td>$10^{-5.8}$</td>
<td>$10^{-6.0}$</td>
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* TCID$_{50}$ is the 50% tissue culture infective dose in mouse embryo cultures.

were detected in cell extracts and in media (Table 1). KVBCG2A cells grew well in serum-free medium but could not be cloned in soft agar or grown from single cells in microtitre plates. Flasks of KVBCG2A cells subcultivated five times, in medium containing 0.5% hyperimmune K virus antiserum whose HI titre was 1:2560, remained positive for K virus V antigen and infectivity.

A second flask, KVBCG1B, also developed islands of cells with altered morphology and density dependence. These cells, like KVBCG2A cells, became the predominant cell type and could be subcultivated rapidly. KVBCG1B cultures contained numerous cells positive for K virus V antigen up to the 15th subcultivation. Numbers of positive cells diminished rapidly thereafter and could not be detected after the 21st subcultivation. Cells at this and subsequent passages were negative for K virus infectivity by virus assay and by inoculation of newborn mice. KVBCG1B cells grew well in serum-free medium, could be grown from single cells in microtitre plates, and had a cloning efficiency of 14% in soft agar. Newborn inbred BALB/c mice inoculated with KVBCG1B cells and immunosuppressed with anti-thymocyte serum (Greenlee, 1979) developed large subcutaneous tumours. Sera from tumour-bearing mice produced nuclear fluorescence typical of papovavirus T antigen staining when used to overlay cells grown from the animals' explanted tumours, parent KVBCG1B cells (Fig. 2), and mouse embryo cells infected with K virus. These sera did not produce nuclear fluorescence when reacted with uninfected mouse embryo cells or cells infected with polyoma virus. KVBCG1B
Short communication

Fig. 3. Southern blot analysis of DNA purified from KVBCG1B cells. Six µg of DNA were cleaved to completion by restriction endonucleases EcoRI (lane 1), BglII (lane 2), XhoI (lane 3) and BamHI (lane 4) and fractionated in agarose gel. After transfer to a nitrocellulose filter, the DNA was hybridized with a nick-translated K virus 32P-DNA probe.

cells did not exhibit nuclear fluorescence when stained with antisera to polyoma virus V or T antigens.

The presence of K virus DNA sequences in KVBCG1B cells was demonstrated by DNA hybridization to a specific 32P-DNA probe (Fig. 3). In this analysis, the cellular DNAs were cleaved to completion with BamHI, XhoI, BglII and EcoRI. The expected cleavage patterns generated by these enzymes from free K virus DNA are as follows (Law et al., 1979): for BamHI, which does not recognize any site in the genome, viral DNA should remain as a circular molecule upon digestion; XhoI cuts K virus DNA at one site and linearizes the circular DNA into a 5 kb fragment; BglII cleaves the DNA into a 2.9 kb and a 2.1 kb fragment; EcoRI cleavage generates three species of 2.1, 2.0 and 0.9 kb. In the present study, analysis of a Southern blot revealed that virus-specific DNA sequences were detectable and that all the expected restriction fragments indicated above were present. Additional bands were also detected, however, indicating that at least a portion of the K virus sequence was integrated into the mouse chromosome. The viral DNA sequences detected as being the expected species may be the cleavage products from some free K virus DNA molecules as well as from the tandemly integrated viral DNA. There appeared to be less than ten genome equivalents of this per diploid genome, and the number of sites of K virus DNA integration into this cell line seemed to be limited.

The present study demonstrates that inoculation of primary cultures of foetal mouse glial cells with K virus results initially in productive infection and subsequently in both persistent infection and cell transformation. Persistent K virus infection of mouse glial cultures (KVBCG2A cells) did not produce c.p.e., despite high titres of viral infectivity in cell
suspensions and media but rather caused cells within these cultures to develop altered morphology and density dependence, increased longevity, and ability to grow in serum-free medium but not in soft agar. Ultrastructural studies still in progress indicate that the initial K virus infection of mouse glial cultures involves oligodendroblasts, astrocytes, neuroblasts and mesenchymal cells but that viral replication during persistent infection is restricted to astrocytes (M. L. Oster-Granite & J. E. Greenlee, unpublished results).

K virus invades the central nervous system during acute infection of suckling mice (Greenlee, 1979), and infectious virus or viral antigen can be detected for as long as 4 months in brains of animals surviving acute infection, despite high titres of circulating antiviral antibody (Greenlee, 1983). The ability of K virus to cause persistent infection in the presence of antiviral antibody in vitro thus parallels the behaviour of this virus in vivo, and is of considerable interest given the ability of JC virus to persist, despite circulating antibody, in brains of human patients with PML (Padgett & Walker, 1983). In the present study, cells exhibiting immunofluorescent staining for K virus T antigen were not more numerous in late-passage KVBCG2A cultures than were cells positive for V antigen. These data would be consistent with maintenance of viral persistence through lytic infection of scattered cells or else with widespread viral persistence unaccompanied, except in rare instances, by expression of early or late viral proteins.

Cell transformation by K virus following inoculation in vitro has not been described previously. Takemoto & Fabisch (1970) reported that lung explants from mice dying of K virus pneumonia gave rise to cell lines which possessed altered morphology and grew well in soft agar. These cells exhibited bright nuclear fluorescence when overlaid with mouse hyperimmune serum to K virus but did not produce infection in newborn mice, indicating restriction of K virus replication. Attempts to produce tumours in mice with these cells were unsuccessful, however. In the present study, inoculation of mouse glial cultures with K virus produced a cell line, KVBCG1B, which possessed biological properties indicative of cell transformation. Although KVBCG1B cells did not contain infectious virus or viral capsid antigen, nucleic acid hybridization studies unequivocally demonstrated the presence of KV DNA sequences in KVBCG1B cells and identified all viral DNA fragments expected from treatment with restriction endonucleases recognizing sites spread among different regions of the viral genome. It would thus appear that major rearrangement and/or deletion of the viral genome had not occurred in this cell line. Southern blot analysis revealed additional bands of flanking sequences which did not co-migrate with any of the expected viral DNA fragments. This observation suggests that cell transformation by K virus, like transformation induced by polyoma, SV40, BK and JC viruses, may be associated with integration of the viral genome into chromosomal DNA of the host cell.

The present study represents the first instance in which antisera specific for K virus T antigen have been developed. K virus T antibody present in sera from KVBCG1B tumour-bearing mice stained cells productively infected and transformed by K virus but did not stain cells productively infected or transformed by polyoma virus, nor did KVBCG1B cells exhibit nuclear fluorescence when stained with antisera to polyoma T or V antigens. These data, which suggest that there is little antigenic similarity between early proteins of K virus and polyoma virus, are consistent with nucleic acid homology studies comparing the genomes of the two agents (Bond et al., 1978; Law et al., 1979) and with previous immunofluorescence studies of K virus-transformed lung cells (Takemoto & Fabisch, 1970; Bond et al., 1978). Additional experiments are planned to characterize K virus T antigen more precisely. The development of cell lines persistently infected and transformed by K virus and the availability of antisera directed against early viral proteins should allow detailed studies to determine the extent to which the biological behaviour of K virus resembles that of polyoma virus, SV40, and human papovaviruses BK and JC.

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Short communication

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