Isolation of a New Strain of Cytomegalovirus from Explanted Normal Skin

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SUMMARY

Forty-three fibroblast cell lines were initiated from normal skin biopsies. One cell line from a patient with Charcot-Marie-Tooth disease (CMT) spontaneously developed c.p.e. suggestive of cytomegalovirus (CMV). Characterization of the virus showed it to be a new strain of CMV and the results suggested that skin fibroblasts from CMT patients may carry latent CMV.

Forty-three fibroblast cell lines were initiated from normal skin biopsies obtained from the side of an incision for a diagnostic nerve biopsy or a similar operative incision elsewhere. Nine of these cell lines were started from nine patients with Charcot-Marie-Tooth disease (CMT), a familial peripheral neuropathy (Dyck & Lambert, 1968) and 34 from control patients with other nerve diseases or patients requiring surgery. Skin samples, minced finely by scalpel, were planted in RPMI-1640 with 20% heat-inactivated foetal calf sera (FCS; Gibco, Grand Island, N.Y.), containing 100 units penicillin and 100 μg streptomycin/ml, in 25 cm² plastic tissue culture flasks and incubated at 37 °C in humidified air with 5% CO₂. All cultures were grown by standard procedures using 0.2% trypsin in saline for passage and 10% FCS for maintenance media.

Unusual initial growth characteristics were observed in all CMT cultures compared to the 34 control cultures, in that primary epithelial colony formation occurred in all of nine CMT patient cultures, whereas 9 out of 34 control cultures (26.5%) showed this cell type. Following the initial growth of epithelial colonies and the persistence of epithelioid cells for several passages, fibroblast cells eventually predominated in all cultures. Fifty thousand cells from eight CMT and five control lines (12th to 15th passage) were tested for growth in 0.3% soft agar (Lang et al. 1974). Small colony formation occurred with four out of eight CMT lines as well as one out of five controls; however subsequent transfer into plastic culture flasks resulted in failure of growth. Population doubling times when fibroblastic were identical in CMT and control cultures (48 ± 12 h). However, periods of poor growth or ‘crisis’ occurred irregularly in the CMT lines in which the cells became enlarged, epithelioid-like and demonstrated decreased density at confluency (0.85 × 10⁶ average CMT cells/25 cm² flask to 1.5 × 10⁵ average control cells/25 cm² flask), but displayed no criss-cross, piled-up or irregular areas to suggest in vitro transformation. At this time the passage history of the CMT cells is within normal life expectancy of in vitro human fibroblasts.

In one cell line originating from a CMT patient (designated CMT-6), several small foci of large refractile cells appeared spontaneously during the sixth passage following a ‘crisis’ period. These foci increased both in number and size with passage and were characteristic of CMV-induced c.p.e. (Glaser et al. 1977). The c.p.e. progressed slowly over a 2 to 3 week period to involve 80 to 100% of the cell monolayer. Haematoxylin and eosin staining of infected monolayers revealed the presence of eosinophilic type A inclusion bodies in the nuclei of infected cells. Further characterization with acridine orange demonstrated apple-green fluorescence indicative of double-stranded nucleic acid in the nuclear and cytoplasmic inclusion bodies of infected cells.
Short communications

Fig. 1. Electron micrograph of a fibroblast infected with agent CMT-6. Nucleus (N) contains numerous unenveloped virus particles (105 nm). Cytoplasm (C) shows virus particles encapsulated (150 nm), many with fibrillar coats (arrows) with and without cores. Dense bodies (DB) with budding virus particles are characteristic of cytomegalovirus.

Five \( \times 10^6 \) infected cells and supernatant from the CMT-6 line were inoculated on to cell cultures originating from different species: Rhesus monkey kidney, mouse embryo tissue culture, Wi-38 (Flow Laboratories, Rockville, Md.), Detroit 550 (American Type Culture Collection CCL 109), rabbit kidney, human neonatal foreskin and several adult human fibroblastic and epithelioid skin lines (initiated in this laboratory). Evidence of virus replication was determined by the appearance of c.p.e. Cultures were observed for periods ranging from 3 weeks for kidney lines to 6 weeks for fibroblastic lines. The CMT-6 isolate replicated only in fibroblast cells of human origin.

Electron microscopic techniques showed numerous intranuclear and cytoplasmic enveloped (150 nm) and unenveloped (105 nm) capsids with a high percentage of coreless forms. Consistent with CMV-type replication were the fibrillar material coating the cytoplasmic enveloped capsids and the dense bodies with budding virus particles found frequently in the cytoplasm (Smith & de Harven, 1973; Fig. 1). Hyperimmune human anti-CMV serum (provided by Dr Ronald Glaser, Ohio State University Department of Medical Microbiology) was used as the primary reagent in the indirect immunofluorescent antibody test to confirm the human origin of the isolate (Glaser et al., 1977). Specific green fluorescence was
Fig. 2. Co-electrophoresis comparison of DNA from agent CMT-6 (on Detroit 550 fibroblasts, labelled as D550) with five other cytomegalovirus (CMV) isolates by cleavage of the DNA with restriction endonucleases (a) EcoRI and Xba and (b) EcoRI and Bam, and separation of the fragments in 1% agarose gels. (a) CMT-6 (D550) is compared with T (Towne CMV isolate) and A, B and C (Dr S. Plotkin’s CMV throat isolates). (b) CMT-6 (D550) is compared to Mj (Dr F. Rapp’s Major CMV isolate).

seen in the nuclear and cytoplasmic inclusions of agent CMT-6-infected cells, but not in un-infected skin cell controls. Furthermore, no staining was seen when CMV-negative serum (also provided by Dr R. Glaser) was used as the primary reagent in agent CMT-6-infected cells or uninfected control cells.

Escherichia coli restriction endonuclease fragmentation analysis of the CMT-6 agent on D550 (Detroit 550 human fibroblasts) and five other CMV isolates was carried out and compared. $^{32}$P-labelled virus DNA of each isolate was purified from extracellular fluid of the virus-infected cells as described previously (Huang et al. 1973). Virus DNA in tris-buffered
saline (0.05 M-tris-HCl, pH 7.4, 0.15 M-NaCl) was digested with restriction endonuclease EcoRI, Xba or Bam in the presence of 0.01 M-MgCl₂ and 0.006 M-mercaptoethanol for 6 h. The DNA fragments were subjected to 1% agarose slab-gel electrophoretic analysis and then autoradiographed in Kodak X-ray film (XR-2) as previously described (Kilpatrick et al. 1975). Comparison of the six CMV isolates showed similarities between them; however, the CMT-6 agent most closely resembled the pattern of the CMV strain Mj, isolated from a child's normal prostate (Rapp et al. 1975) (Fig. 2).

Virus rescue attempts, carried out with the portion of CMT-6 cell line that had not shown infection, the remaining eight CMT fibroblast lines and eight randomly selected control lines (passages 15 to 20) were unsuccessful. Rescue methods included: (i) co-cultivation with permissive Wi-38 (human embryonic lung) or (ii) treatment with one of the following compounds: IdUrd, 25 μg/ml for 4 days; dexamethasone, 1 μg/ml for 6 days; pokeweed mitogen, 1% for 7 days; E. coli lipopolysaccharide, 12 μg/ml for 5 days; concanavalin A, 30 μg/ml for 3 days (all chemicals from Sigma, St Louis, Mo.) or sequential temperature extremes (40 °C for 3 days followed by 25 °C for 7 days).

Exogenous contamination of the CMT-6 cell lines was unlikely since 42 other fibroblast lines, carried simultaneously under identical cultural conditions, remained negative, ruling out infection from media or supplements. Furthermore, the two individuals handling the cell lines had low antibody titres to CMV (< 1:8) and negative urine cultures for virus taken 3 weeks and 3 months after the initial appearance of c.p.e. No virus was used in this laboratory and no tumour-derived cell lines were maintained. The appearance of c.p.e. in line CMT-6 occurred 6 months after explanting the original biopsy, thereby excluding or minimizing the chance of a 'passenger' contaminant. All cultures were consistently negative for mycoplasma growth (Mycoplasma Testing Agar and Broth, Microbiological Associates, Bethesda, Md.).

Primary epithelial colony growth and similar 'crisis' periods, as seen in our CMT explants, have been reported in prostatic tissue explants (Webber, 1976) and Kaposi sarcoma skin cultures (Giraldo et al. 1972). In both cases an association with CMV was determined (Giraldo et al. 1975; Glaser et al. 1977). The growth of four out of eight CMT lines in soft agar, while usually observed with tumour cells or cells transformed in vitro by chemical carcinogens or oncogenic viruses, has been documented for human cells inoculated with irradiated CMV (Lang et al. 1974).

Host-range specificity, immunofluorescent electron microscopic and restriction endonuclease fragmentation analysis of the DNA from virus agent CMT-6 provide evidence for the isolation of a new strain of cytomegalovirus. Its recovery from one CMT fibroblast line and the unusual growth characteristics observed in several of the other CMT lines suggest the possibility of CMV latency in some of these cells (Michelson-Fiske, 1977).

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