Characterization of Tumor Cell Lines Derived from Murine Gammaherpesvirus-68-Infected Mice

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Cell lines were derived from mice with murine gammaherpesvirus-68 (MHV-68)-associated lymphoproliferative disease. Four were of an ambiguous phenotype and were MHV-68 negative. One, S11, was a B lymphocyte that contained MHV-68 genomes in both linear and episomal forms and released virus. The line was clonable and grew into tumors in nude mice. This is the first naturally occurring MHV-68-positive B-cell line to be generated, and it will be an invaluable tool for the study of MHV-68 latency.

Murine gammaherpesvirus-68 (MHV-68) provides an amenable small-animal model for the study of gammaherpesvirus pathogenesis. MHV-68 establishes a latent infection in B lymphocytes following an acute respiratory infection (1a, 14–16). Previous results from this laboratory have shown that at late times after infection (>6 months) some 10% of mice developed lymphoproliferative disease and after treatment with cyclosplorin A the proportion rose to 60%. A high proportion (50%) of affected mice displayed high-grade lymphomas (13). These lymphomas were usually associated with the spleen or mesenteric lymph nodes; however, lung, liver, and kidney tissues were also affected.

In terms of cellular transformation the most thoroughly studied gammaherpesviruses are Epstein-Barr virus (EBV) and herpesvirus saimiri. Cell lines derived from EBV-, herpesvirus saimiri-, and herpesvirus ateles-induced tumors have been extremely useful in the study of virus latency and the discovery of virus genes responsible for cellular transformation.

This paper reports the derivation of five cell lines from MHV-68-infected mice with lymphoproliferative disease, one of which was infected with MHV-68. The nature of the infection in this cell line was investigated and was found to be very similar to that reported for other gammaherpesvirus-transformed cell lines.

Derivation and phenotype of lymphoma cell lines. Lines were generated from BALB/c mice which had been infected with MHV-68 for >1 year and showed evidence of lymphoma development in the spleen and/or lymph nodes. Tumor cells were purified by centrifugation over Histopaque-1077 (Sigma-Aldrich) initially cultured at a density of 2×10^6 cells per ml in RPMI medium plus 10% fetal calf serum. When lines began to grow, they were passaged at 1×10^5 to 2×10^5 cells per ml.

Lines BLN, SPL, and MLN were derived from mice which were treated with 1 mg of cyclosporin A (a gift from D. White, Addenbrookes Hospital, Cambridge, United Kingdom) in olive oil on days 3, 7, 14, 21, and 28 following infection. BLN and SPL were from the basal lymph node and spleen of one animal, and MLN was from the mesenteric lymph node of a second animal. Lines S11 and S31 were both derived from spleen cell cultures, with S31 coming from a thymectomized CD8-de-

pleted mouse treated with the antiherpesvirus compound 2'-deoxy-5-ethyl-beta-4'-thiouridine (C9) (9). This compound has been shown to be highly effective in inhibiting MHV-68 replication (1).

Each line was stained with a panel of monoclonal antibodies specific for lymphocyte surface markers, and the presence of these markers was measured by cytofluorimetric analysis using a FACScan device (Becton Dickinson) as described previously (17). All antibodies—except biotinylated anti-immunoglobulin M (IgM) (Sera-lab, Sussex, United Kingdom); biotinylated (Fab')₂ anti-mouse Ig and fluorescein isothiocyanate-conjugated anti-rabbit Ig (Dako Ltd., High Wycombe, United Kingdom); fluorescein isothiocyanate-conjugated anti-rat Ig (Serotec, Oxford, United Kingdom); and anti-CD3 and anti-CD5, which were hybridoma supernatants from the lines KT3-1.1 and YTS121.5.2, respectively (gifts from S. Cobbold, University of Oxford)—and fluorescein isothiocyanate-conjugated streptavidin were purchased from Pharmingen (San Diego, Calif.). All lines expressed major histocompatibility complex class I MHC-I and intercellular adhesion molecule 1 (ICAM-1) (Table 1), whereas none expressed CD3, a marker restricted to T lymphocytes. Line S11 also expressed IgM on the cell surface, in addition to major histocompatibility complex class II and a very low level of the B-cell-specific marker B220. Line S31 expressed a very low level of B220 but was negative for surface IgM, and lines MLN, SPL, and BLN were negative for all other surface markers. The presence of intracellular Ig was also tested by immunocytochemistry; the only positive line was S11. Thus, S11 was of the B-lymphocyte lineage, whereas the phenotypes of the other lines remained ambiguous. Lines MLN, BLN, and SPL grew as a layer of adherent single lymphoblastoid-type cells; S11 cells had a similar morphology but grew in small clumps of approximately 10 to 12 cells with some adherent cells, a few of which sent out elongated processes. S31 cells were similar in size to resting lymphocytes and grew in large balls.

Cytogenetic analysis of the lines. Lines BLN, MLN, SPL, and S11 had multiple chromosomal duplications and translocations. Line S31 contained 41 to 42 chromosomes in comparison with the normal diploid complement of 40 chromosomes.

Presence of MHV-68 in tumor cell lines. High-molecular-weight DNA was prepared from each cell line and used as a substrate for PCRs with primers specific for the MHV-68 gp150 gene as described previously (12). This analysis showed that S11 was the only line carrying the MHV-68 genome. This line was investigated in greater detail. The PCR was made

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TABLE 1. Phenotypes of tumor cell lines[a]

Antigen	Fluorescence intensity (arbitrary units) in:				
	S11	S31	BLN	MLN	SPL
CD3	1	0	0	0	2
IgM	26	0	0	0	0
Intracellular Ig[b]	+	−	−	−	−
B220 (CD45R)	6	6	2	1	3
CD5	1	1	0	1	1
MHC-I	39	50	35	25	32
MHC-II	39	43	8	0	5
ICAM-1	21	54	36	36	39
MHV-68[c]	+	−	−	−	−

[a] Surface antigen expression was measured by flow cytometry. Values shown are mean fluorescence intensities after subtraction of background fluorescence (second-step reagent alone).

[b] +, positive staining by immunofluorescence; −, no staining observed.

[c] +, DNA samples positive by PCR; −, DNA samples negative by PCR.

quantitative by the use of a competitive template that contained the same primer binding sites but was different in size. Such a mutant competitor template was made by first cloning the 480-bp gp150 PCR product into the vector pUC19. A 100-bp fragment was then deleted from the insert by cutting plasmid DNA with *Ava*I, religating, and recloning. This generated a plasmid with an insert which contained the binding sites for the gp150 primers but which, when amplified, gave a product which was 100 bp smaller than that produced by amplification of viral DNA. A series of PCRs were then performed with the same amount of S11 DNA but spiked with a dilution series of defined quantities of mutant competitor template. The relative amounts of mutant competitor and target sequence were analyzed by densitometry, and the amount of S11 DNA present was calculated by construction of a standard curve exactly as described previously (6, 10). By using this technique on DNA derived from S11 cells which had been treated for 2 weeks with C9 to eliminate traces of DNA associated with productive replication, it was established that, on average, each cell contained 40 copies of the virus genome.

A proportion of S11 cells produced virus particles, as cocultivation with permissive BHK cells (14) produced infective centers which stained positive with anti-MHV-68 rabbit hyperimmune serum. The line became more lytic with passage in vitro; 2 to 4% of the cells gave rise to infective centers after 12 weeks in culture, with the level rising to 6 to 7% after 5 months and 20 to 30% after 9 months. Free virus was detectable in the culture supernatant and in cell lysates.

To determine whether it was possible to increase the number of lytically infected cells in the culture, replicate cultures were treated with either 20 ng of phorbol 12-myristate 13-acetate (TPA) per ml dissolved in dimethyl sulfoxide or an equal volume of dimethyl sulfoxide alone and incubated for 48 h. Immunofluorescent staining of cell smears with rabbit hyperimmune anti-MHV-68 serum revealed that 3.2% of the control cells were positive for virus antigen compared with 10.4% of the TPA-treated cells. Thus, in a fashion analogous to that observed with EBV (19), TPA could reactivate otherwise latently infected cells within the culture.

The integrity of the virus genome in the S11 line was tested by preparing virion DNA from productively infected BHK cells and comparing this DNA with that of the parental strain (g2.4) by restriction enzyme analysis using the enzymes *Bam*HI, *Eco*RI, and *Hin*dIII. This analysis revealed no observable deletions in S11 virus relative to the parental strain, in contrast to

deletions reported for other gammaherpesviruses isolated from latently infected lines (7).

Antiherpesvirus drug treatment of the S11 line. To determine the effect of antiherpesvirus drug treatment on the MHV-68-infected S11 line, cells were cultured in the presence of 1 μg of C9 per ml. At 4 or 19 days posttreatment cells were cocultivated with BHK cells for 5 days and the number of infective centers was enumerated. Cell lysates, obtained by three cycles of freeze-thaw in liquid nitrogen, were also cocultivated to determine the level of free (i.e., nonlatent) cell-associated virus. The results are shown in Fig. 1. C9 treatment reduced the level of free virus to undetectable levels, whereas the cells remained latently infected as evidenced by the fact that 5 to 6% reactivated to give rise to infective centers. After withdrawal of the drug, free virus was again detectable.

Latent infection in the S11 line. Studies with related gammaherpesviruses have shown that viral genomes are in a linear form during productive replication but an episomal form during latency. To determine the state of the virus genome in the S11 line, cells were subjected to electrophoresis according to the method of Gardella et al. (5), which resolves episomal and linear genomes. A blot of the gel was probed with a ^{32}P-labelled probe specific for the MHV-68 gL homolog as described previously (11), and the relative proportions of linear and episomal DNA were estimated by using Quantiscan densitometric software (Biosoft, Cambridge, United Kingdom). As shown in Fig. 2, the S11 line contained both episomal and linear forms of the MHV-68 genome, in a ratio of 1:3.0. Pretreatment of the S11 cells with 100 μM acyclovir for 48 h resulted in an increase in the ratio of circular genomes to linear genomes to 1:2.3. Conversely, pretreatment with 20 ng of TPA per ml decreased the circular-to-linear ratio to 1:11.6, consistent with reactivation of latent virus from the line.

We were concerned that the S11 line may have been a carrier culture in which only a proportion of the cells were infected. To address this question, the line was washed to remove free virus and then cloned by limiting dilution at 0.3 or 1 cell per well. Eleven clones, all of which were positive for virus genomes by PCR, were derived in this way, demonstrating that all cells in the culture were MHV-68 infected.

Growth of the S11 line in nude mice. Two groups of six BALB/c nude mice received either 10^6 or 10^7 S11 cells subcutaneously in the lower left flank and were observed over a period of 1 month for tumor development. For the mice receiving 10^7 cells, three of six developed solid tumors in situ,
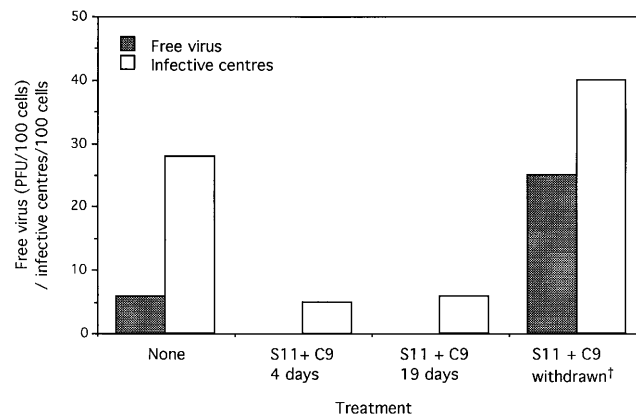

FIG. 1. Numbers of infective centers and free (i.e., nonlatent) virus PFU after treatment of the S11 line with C9. †, cells were cultured in the presence of 1 μg of C9 per ml for 15 days, and then the drug was withdrawn for 4 days.
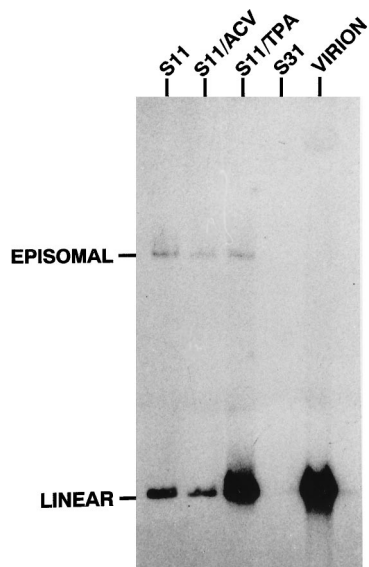
FIG. 2. Gardella gel analysis of cell lines and MHV-68. Cells (2×10^6) or MHV-68 virions (100 μl of stock at 10^7 PFU/ml) were loaded into the wells of a horizontal Gardella agarose gel, electrophoresed, and blotted, and blots were probed with an MHV-68 gL homolog probe. S11 tumor cells were loaded either without treatment or after treatment with acyclovir (ACV) or TPA for 48 h as indicated above the tracks. The relative positions of linear and circular virion DNA are indicated on the left.

compared with one of six for the group receiving 10^6 cells. Subcutaneous injection of S11 cells into euthymic BALB/c mice did not result in tumor development.

This report represents the first study of a naturally infected B-cell line explanted from an MHV-68-infected mouse. MHV-68 behaved in a way similar to that of other gammaherpesviruses during a latent infection in the S11 line, the genome persisted in an episomal form, and the culture could not be cured with an antiherpesvirus drug. When injected into athymic nude mice, S11 cells gave rise to tumors, and it was possible to grow clones from single cells of the S11 line. These last two properties suggest that the line is more reminiscent of Burkitt's lymphoma cells than it is of lymphoblastoid cell lines derived from in vitro infection (8), although the S11 cells did have an activated phenotype and grew as clumps.

Only one of five cell lines from MHV-68-infected mice carried the virus genome. This may indicate a role for the virus in tumor initiation, but it does not appear to be necessary for continued tumor cell growth. This would not be unusual, as EBV-related Burkitt's lymphoma is believed to require other cofactors, such as malaria infection, for tumor development (2, 3, 18).

This reagent should prove invaluable in the hunt for virus genes expressed during latent infection and also as a target cell for virus-specific cytotoxic T lymphocytes. In addition it may provide information on transformation processes used by human gammaherpesviruses such as the newly discovered human herpesvirus 8 (4) and EBV.

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