Journal of General Virology (1999), **80**, 2729–2736. Printed in Great Britain

Kinetic and phenotypic changes in murine lymphocytes infected with murine gammaherpesvirus-68 *in vitro*

Bernadette M. Dutia, James P. Stewart, Robert A. E. Clayton, Heather Dyson and Anthony A. Nash

Department of Veterinary Pathology, University of Edinburgh, Summerhall EH9 1QH, UK

Primary infection with murine gammaherpesvirus-68 (MHV-68), as with other members of the gammaherpesvirus subfamily, is characterized by a lymphoproliferative phase. MHV-68 causes acute splenomegaly and an infectious mononucleosis-like syndrome in which there is expansion of the $CD8^+$ T cell subset. In long-term infections, MHV-68 is associated with lymphoma development. In order to elucidate the mechanisms underlying the proliferative processes, the events following infection of murine splenocytes or purified murine B lymphocytes *in vitro* have been examined. MHV-68 infection prolonged the viability of murine splenocytes and stimulated cellular proliferation. Unlike Epstein–Barr virus and herpesvirus saimiri, MHV-68 did not cause growth transformation. Growth transformation did not occur even when cells with a predisposition to transformation were infected or when culture conditions were selected to enhance the viability of the cells. Following MHV-68 infection, the latency-associated viral tRNAs were transcribed. However, transcription of the other known latency-associated gene, M2, was not observed. In addition, there was no evidence of productive virus replication either by staining with antibodies specific for late virus antigens or by *in situ* hybridization for early and late mRNAs. In contrast to Epstein–Barr virus- and herpesvirus saimiri-infected lymphocytes, where episomal genomes are seen, Gardella gel analysis indicated that the primary lymphocytes infected by MHV-68 *in vitro* contained only linear virus DNA. This DNA was nuclease sensitive, indicating that, while MHV-68 was efficiently uncoated, its circularization *in vitro* was extremely inefficient. These results are discussed in terms of the host–virus interaction.

Introduction

Members of the gammaherpesvirus subfamily are characterized by their association with lymphoproliferation and tumorigenesis. The human herpesvirus Epstein–Barr virus (EBV) causes lymphoproliferative disease as well as nasopharyngeal carcinoma and Burkitt's lymphoma, while human herpesvirus-8 (HHV-8) (also called Kaposi's sarcoma-associated herpesvirus) is associated with body-cavity lymphomas and Kaposi's sarcoma (Rickinson & Kieff, 1996; Schulz, 1998). Animal gammaherpesviruses, too, show similar associations: herpesvirus saimiri (HVS) with T cell lymphomas and alcelaphine herpesvirus-1 with malignant catarrhal fever (Roizmann *et al.*, 1992). The murine gammaherpesvirus MHV-68 has been established as a working model for gammaherpesviruses both *in vivo* and *in vitro* (Nash *et al.*, 1996). After intranasal infection, MHV-68 replicates in the lungs prior to establishing a latent infection in B lymphocytes in the spleen (Sunil-Chandra *et al.*, 1992*a*, *b*). Latent virus is also detectable in lung epithelial cells and macrophages (Stewart *et al.*, 1998; Weck *et al.*, 1999). During the acute phase of infection, MHV-68 causes a $CD4^+$ T cell-dependent, transient splenomegaly involving proliferation of both B and T lymphocytes (Ehtisham *et al.*, 1993; Usherwood *et al.*, 1996*a*), followed by a mononucleosis consisting predominantly of an expansion of Vβ4 T cell receptor-expressing $CD8^+$ T cells (Tripp *et al.*, 1997). In the long term, infection is associated with the development of lymphomas, although the exact relationship between MHV-68 and these tumours is still unclear (Sunil-Chandra *et al.*, 1994).

The association of EBV with malignant disease *in vivo* is reflected in the ability of the virus to effect growth transformation of human B cells *in vitro*. Resting B cells are the primary target for growth transformation *in vitro* but the virus can also transform progenitor B lymphocytes, pre-B lympho-

Author for correspondence: Bernadette Dutia.
Fax +44 131 650 6511. e-mail B.M.Dutia@ed.ac.uk

0001-6358

cytes and cells with fully rearranged and mature immunoglobulin chains (Ernberg *et al.*, 1987). Growth transformation of marmoset and human T cells by HVS is readily achieved (Biesinger *et al.*, 1992; Schirm *et al.*, 1984; Fickenscher & Fleckenstein, 1998) and recent experiments have shown that HHV-8 can transform primary human endothelial cells *in vitro* (Flore *et al.*, 1998).

For EBV, the process of growth transformation *in vitro* has been widely studied and is the primary experimental system used to identify the viral proteins involved in transformation and to characterize their functions (Raab-Traub, 1996). The initial events in the process of infection of primary B cells are mediated by the binding of the virus to its receptor and mirror the changes that occur during mitogen- or antigen-induced activation (Gordon *et al.*, 1986; Sinclair & Farrell, 1995). Within 12–16 h of infection, circularized EBV genomes are detected and virus gene transcription commences (Hurley & Thorley-Lawson, 1988). EBNA2 and EBNA-LP are the first genes transcribed. By 32 h post-infection (p.i.), transcription of the nine genes expressed in EBV-growth-transformed B cells can be detected, while transcription of the EBER RNAs lags by 24 h and does not reach substantial levels until 70 h p.i. (Alfieri *et al.*, 1991). Over the next 5 days, cell proliferation begins and colonies can be detected from 1 week p.i. (Stuart *et al.*, 1995).

In order to further our understanding of the biology of MHV-68, we have studied the effect of infection of murine splenocytes and purified B lymphocytes *in vitro*.

Methods

■ **Mice.** Female BALB/c mice were purchased from B & K Universal Ltd (Grimston, Hull, UK) and used when more than 6 weeks of age. Homozygous $p53^{-/-}$ and heterozygous $Rb\text{-}1^{+/-}$ mice (Clarke *et al.*, 1992, 1993) were a generous gift from A. Clarke, Department of Pathology, University of Edinburgh, UK.

■ **Virus and cells.** MHV-68 was originally obtained from D. Blaskovic (Blaskovic *et al.*, 1980). Working stocks of MHV-68 were prepared by infection of BHK-21 cells with MHV-68 clone g2.4 (Efstathiou *et al.*, 1990) at low multiplicity (0·001 p.f.u. per cell) as described previously (Sunil-Chandra *et al.*, 1992 *a*). S11 is a B cell tumour line derived from a BALB/c mouse and was grown as described previously (Usherwood *et al.*, 1996 *b*). The CD40L-expressing cell line K47 was a generous gift from A. Schhimpl, Institut für Virologie und Immunobiologie, Universität Würzburg, Würzburg, Germany.

■ **Purification of B cells.** B lymphocytes were purified from total splenocytes by negative selection by using CD43 MACS beads (Miltenyi Biotec) according to the manufacturer's directions. This method routinely gave preparations that were 95% pure.

■ ***In vitro* infection of lymphocytes.** Cells were teased out of spleens and mononuclear lymphocytes were purified by centrifugation on Ficoll-Hypaque gradients (Histopaque 1077, Sigma). Lymphocytes were washed extensively with RPMI containing 10% foetal calf serum, 50 μM 2-mercaptoethanol, 2 mM glutamine, 100 μg/ml streptomycin, 100 U/ml penicillin and 2 μg/ml fungizone, resuspended at a concentration of 2×10^7 cells/ml and infected with appropriate amounts of virus or mock-infected with BHK cell lysate by incubating on a shaker at 37 °C for 1 h. Cells were diluted to 2×10^6 cells/ml, plated in 24 well tissue culture plates and incubated at 37 °C in a CO_2 incubator for the required time. For long-term experiments, cell were fed weekly with fresh medium and cytokines as described in Results.

■ ***In vitro* survival of lymphocytes.** Lymphocytes were purified, infected at varying m.o.i. and 2 ml samples containing 2×10^6 cells/ml were plated in triplicate. Cells were resuspended thoroughly with a pipette prior to counting. On days 0 and 1 p.i., 50 μl samples were stained with 1% trypan blue and duplicate counts were made of live cells. At later time-points, cells were concentrated 10–30-fold by centrifugation prior to counting to ensure adequate numbers of viable cells for accurate counting.

■ **Lymphocyte proliferation.** Lymphocyte proliferation was measured by using the Biotrak cell proliferation ELISA system 2 (Amersham). B lymphocytes were purified by positive selection with rat anti-mouse CD19 antibody (Pharmingen) and goat anti-rat MACS microbeads according to the manufacturer's instructions. Infected and mock-infected cells were plated at 10^7 cells/ml in 96 well flat-bottomed plates, 100 μl per well. After 72 h, 5-bromo-2′-deoxyuridine (BrdU) was added to a final concentration of 20 μM and cells were cultured for a further 24 h. Cells were then pelleted by centrifugation, the medium was removed and the plates were dried at 60 °C for 1 h. The plates were fixed and stained according to the manufacturer's instructions with the following modifications: fixation time was increased to 1 h, incubation with anti-BrdU antibody was carried out at 37 °C rather than at room temperature and the antibody concentration was increased 4-fold.

■ ***In situ* hybridization.** Non-radioactive *in situ* hybridizations were carried out on MHV-68-infected and mock-infected lymphocytes 72 h p.i. essentially as described previously (Stewart *et al.*, 1998). After recovery of viable lymphocytes by centrifugation over Ficoll-Hypaque, cells were washed twice in PBS, fixed in PLP (75 mM L-lysine hydrochloride, 37·5 mM sodium phosphate buffer, pH 7·4, 2% paraformaldehyde, 10 mM sodium metaperiodate) for 5 min, washed once more and then resuspended in PBS at a concentration of 10^6 cells/ml. Cells (100 μl) were transferred onto Biobond-treated slides by using a cytocentrifuge (Shandon) and fixed again in PLP. S11 cells for use as positive controls were treated similarly except that the cells were assumed to be 95% viable. Probes were digoxigenin-labelled RNAs generated with an RNA-labelling kit (Boehringer Mannheim) and were detected by using a combination of alkaline phosphatase-conjugated anti-digoxigenin antibody and BCIP/NBT tablets (Sigma), followed by counter-staining with neutral red. Sense and anti-sense transcripts spanning MHV-68 tRNAs 1–4 were generated from pEH1.4 (Bowden *et al.*, 1997). The anti-sense probe for the overlapping thymidine kinase/glycoprotein H (tk/gH) mRNAs has been described previously (Stewart *et al.*, 1998). The M2 probe was generated from a DNA fragment containing the complete ORF that had been cloned into pKS (−) (Stratagene).

■ **Gardella gel analysis.** Viable cells were recovered by centrifugation over Ficoll-Hypaque. S11 cells were harvested from exponentially growing cultures. Gardella gel electrophoresis was performed in a horizontal format as described previously (Decker *et al.*, 1996 *a*). Southern blots were hybridized with ^{32}P-labelled probes directed against MHV-68 sequences. DNase I digestion was performed as described previously (Jamieson *et al.*, 1995).

Results

Infection of primary murine lymphocytes with MHV-68

To assess the ability of MHV-68 to initiate growth transformation, primary murine lymphocytes were infected

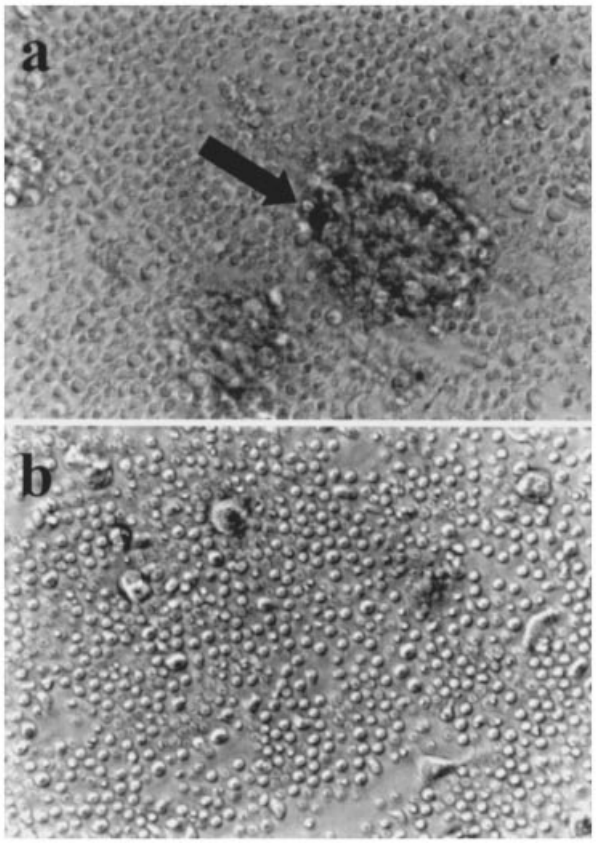

Fig. 1. Cultured BALB/c splenocytes 5 days after infection with 5 p.f.u. MHV-68 per cell (*a*) or mock-infection (*b*). Arrow indicates clump of enlarged cells. Magnification × 300.

with a variety of multiplicities of MHV-68 and cultured *in vitro* as described in Methods. Fig. 1 shows lymphocyte cultures 4 days after virus infection or mock infection. Cultured infected cells contained clumps of enlarged cells that were not present in mock-infected cell cultures. These cells were not growth-transformed and subsequently died, although it was clear that MHV-68 infection caused phenotypic changes in infected lymphocytes.

To quantify these changes, we examined cell viability and proliferation in infected and mock-infected lymphocyte cultures. Cell viability was measured by trypan blue exclusion. Fig. 2 shows that lymphocytes infected with MHV-68 had a better rate of survival up to 7 days p.i. The increased viability was measurable 4 days p.i. This effect was independent of productive virus replication, since we could not detect any significant increase in infectious virus titre in the period 1–5 days p.i. (data not shown). Also, it occurred in the presence of 2′-deoxy-5-ethyl *β*-4′-thiouridine (4′-S-EtdU). This drug has been shown to be highly effective in blocking productive replication both *in vivo* and *in vitro* (Stewart *et al.*, 1998; A. Barnes & A. A. Nash, unpublished observations).

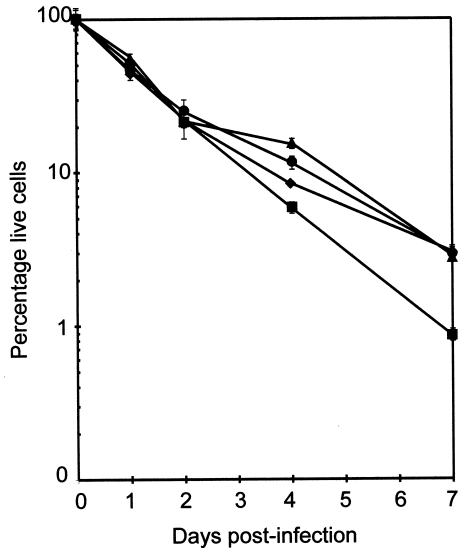

Fig. 2. Viability of mock-infected or MHV-68-infected BALB/c splenocytes cultured *in vitro*. ■, Mock-infected; ◆, 1·0 p.f.u. per cell; ●, 2·5 p.f.u. per cell; ▲, 2·5 p.f.u. per cell cultured with 4′-S-EtdU. Points are means of triplicate counts on duplicate wells. Cell viability was measured by trypan-blue exclusion.

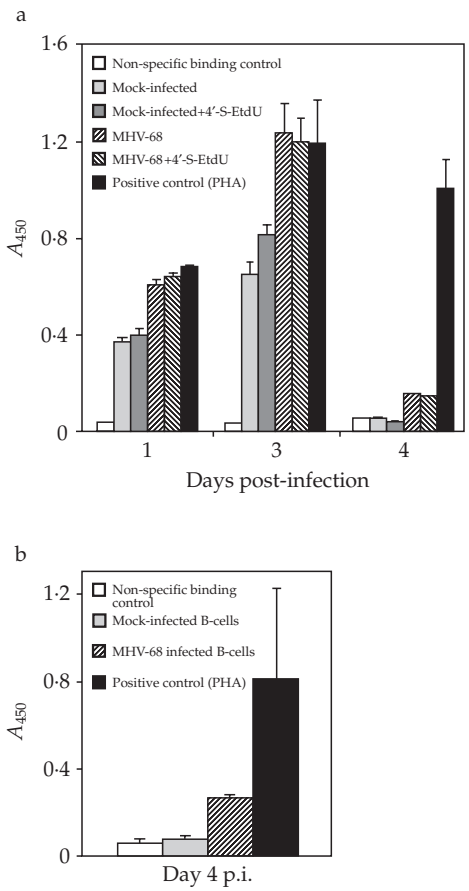

Fig. 3. Proliferation of murine splenocytes cultures *in vitro* after mock-infection or infection with 2·5 p.f.u. MHV-68 per cell, measured by BrdU-based cell-proliferation ELISA. (*a*) Total splenocytes; (*b*) positively selected B lymphocytes.

To address the question of whether the enhanced survival of cultures was due to prolonged survival of infected cells or to cellular proliferation, DNA synthesis was measured by a cell proliferation ELISA. Fig. 3 shows proliferation over 24 h periods from 1 to 4 days p.i. Enhanced proliferation of infected cultures could be seen as early as 24 h, and DNA synthesis was consistently higher in infected than in mock-infected cultures throughout the experiment. The increase in DNA synthesis was seen in cultures treated with 4′-S-EtdU, indicating that cellular DNA synthesis and not productive replication of the virus was being measured. This experiment also shows that cellular proliferation is independent of virus replication and the synthesis of late virus proteins.

Transcription of MHV-68 in lymphocytes infected *in vitro*

Our experiments with infection *in vitro* in the presence of 4′-S-EtdU suggested that productive virus replication did not play a role in the enhanced survival or proliferation of infected cell cultures. To assess further the role of MHV-68 in this process, we investigated virus gene expression by immunohistochemical staining and *in situ* hybridization for known latency- and lytic cycle-associated genes. By immunohistochemical analysis of infected lymphocyte cultures with polyclonal anti-MHV-68 antiserum, we could find no evidence for expression of virus early and late antigens, although this technique readily detected the 1–2% of cells that supported productive virus replication in the S11 tumour cell line (data not shown).

As it appeared that the virus was not expressing lytic cycle-associated antigens in infected cultures, we investigated virus latent gene expression by RNA *in situ* hybridization. The first set of probes detected the transcription of the MHV-68-encoded, tRNA-like molecules. These unique tRNA molecules are transcribed from an RNA polymerase III promoter and are found in both lytically and latently infected cells (Bowden *et al.*, 1997). The second probe was directed against the M2 gene, which is transcribed only in latently infected cells (Husain *et al.*, 1999). Both these probes show similar staining of the S11 tumour cell line (Husain *et al.*, 1999). The third probe was designed to detect the overlapping transcript that detects both the early thymidine kinase mRNA and the late gH message (tk/gH). Fig. 4 shows results of *in situ* hybridization carried out on purified B cells 3 days p.i. The tRNA anti-sense probe detected transcripts in 90% of cells in the infected B cells (Fig. 4*a*). No viral transcripts were detected in these cells with the M2 or tk/gH probes (Fig. 4*c–d*), although both these were positive on the S11 cell line (Fig. 4*g–h*). No positive signal was obtained with the tRNA sense probe on the infected cells (Fig. 4*b*) or with any probe on mock-infected cells (Fig. 4*e–f*). The tk/gH probe confirmed the results obtained with the polyclonal anti-MHV-68 antiserum, that no early or late virus gene expression was occurring in the infected cultures. Similar results were obtained from cultures infected for 2 or 4 days.

Conformation of virus genome in *in vitro* infections

Studies on infection of lymphocytes with EBV *in vitro* have shown that circularization of the virus genome is an early event after infection and that the virus genome is maintained as extrachromosomal, covalently closed circles (episomes) in growth-transformed cells (Hurley & Thorley-Lawson, 1988; Alfieri *et al.*, 1991). This circularization can be readily detected by Gardella gel analysis (Gardella *et al.*, 1984). Circularization also appears to be a prerequisite for herpesvirus DNA replication (Roizman & Sears, 1996). We therefore investigated the form of the virus genome present in total splenocytes, purified B cells and non-B cells infected with MHV-68 *in vitro* (Fig. 5).

Fig. 5(*a*) shows that only linear virus genome was detectable in infected splenocyte cultures. There was no evidence of episomal virus DNA. This contrasts with the S11 tumour cell line where, as shown previously (Usherwood *et al.*, 1996*b*), we could readily detect both linear and episomal forms. It therefore appeared that significant amounts of circularized MHV-68 genome were not present in cells infected *in vitro*, at least within the limits of our assay.

To eliminate the possibility that the linear DNA was not indicative of a failure to circularize but rather a failure of virus capsids to uncoat, we examined the susceptibility of nuclear DNA to DNase I treatment. Encapsidated DNA is resistant to DNase I, whereas uncoated, productively replicating or episomal DNA should be susceptible. In this experiment, we examined four lymphocyte cultures. These included total murine splenocytes (infected or mock-infected), lymphocytes lacking the CD43 cell-surface marker (resting B cells) and CD43-positive cells (T lymphocytes, macrophages and activated B cells). Nuclei from these cells and from the S11 cell line were analysed by using Gardella gels before (untreated) or after treatment with DNase I (Fig. 5*b*). Only linear virus DNA was detected in all the untreated, *in vitro*-infected nuclei. Thus, the virus behaved the same in purified B cells as it did in total lymphocytes. In addition, the virus was able to infect the CD43-positive population readily, indicating that the virus was infecting cell types other than resting B cells. After treatment of nuclei with DNase I, linear genome was no longer present in the *in vitro*-infected cell cultures, whereas, in the S11 cell line, while episomal DNA was absent after DNase I treatment, linear DNA was still present and hence nuclease resistant. This shows that, in this experiment, naked DNA was readily digested whereas the encapsidated DNA present in the 1–2% of S11 cells that support productive virus replication was nuclease resistant. The resistance of encapsidated virus DNA to nuclease digestion in our assay was further confirmed by similar analysis after spiking mock-infected splenocytes with intact virus (results not shown). This experiment therefore

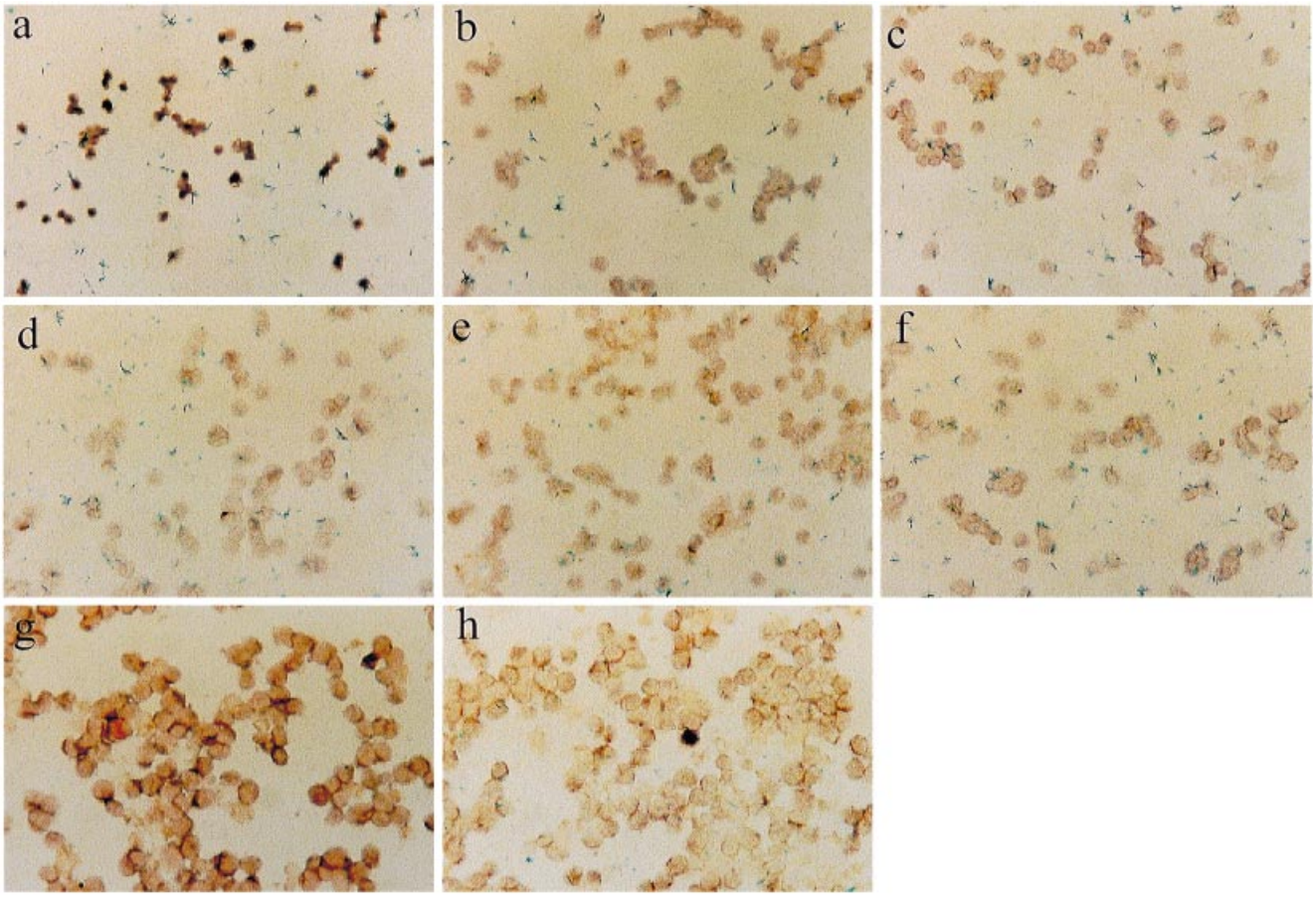

Fig. 4. *In situ* hybridization to detect expression of virus genes in B cells infected *in vitro*. Viable cells were harvested 3 days after infection with 3 p.f.u. MHV-68 per cell (*a*–*d*) or mock-infection (*e*, *f*) and probed with tRNA anti-sense (*a*, *e*), tRNA sense (*b*), M2 anti-sense (*c*, *f*) or tk/gH anti-sense (*d*) probes. (*g*)–(*h*) Positive controls for the M2 and tk/gH anti-sense probes showing positive staining of the S11 cell line with M2 anti-sense (*g*) and tk/gH anti-sense (*h*) probes. Magnification ×275.

confirms that MHV-68 was infecting cultured lymphocytes and that virus DNA was uncoated but that it failed to circularize to any significant extent.

Effect of culture conditions on growth-transforming potential of MHV-68

Although initial experiments showed that MHV-68 did not readily elicit growth transformation of murine lymphocytes, it remained possible that the culture conditions used in these experiments were not optimal. Primary murine lymphocyte cultures have a very limited half-life in the absence of growth factors and activation signals. In an attempt to elicit growth transformation, we designed culture conditions to prolong the viability of the cells and to provide growth signals that might normally be present *in vivo*. Modifications to the growth medium that were used included addition of murine IL-2, IL-4 or IL-10, addition of mitogens such as phytohaemagglutinin (PHA) and addition of conditioned medium from the tumour cell line S11 or from mitogen-stimulated lymphocytes. Infected lymphocytes cultures were plated onto irradiated, CD40L-expressing fibroblasts to provide viability signals or onto irradiated murine splenocytes. We also investigated the possible requirement for activation prior to infection by activating cells with PHA and infecting after the onset of cellular activation. The growth of infected cultures was prolonged under all of these conditions, but we were still unable to demonstrate growth transformation.

To investigate whether the potential of MHV-68 to initiate growth transformation was dependent on predisposing factors in the cell phenotype, we infected splenocytes from $p53^{-/-}$ and $Rb\text{-}1^{+/-}$ mice. Mice with either of these phenotypes have a predisposition to tumorigenesis, $p53^{-/-}$ because of defects in normal induction of apoptosis and $Rb\text{-}1^{+/-}$ because of defects in cell cycle control (Clarke, 1995). Use of these cells did not lead to the derivation of cell lines. We therefore surmised that, although MHV-68 initially affected the phenotype of infected lymphocytes *in vitro*, it was not capable of initiating growth transformation of these cells.

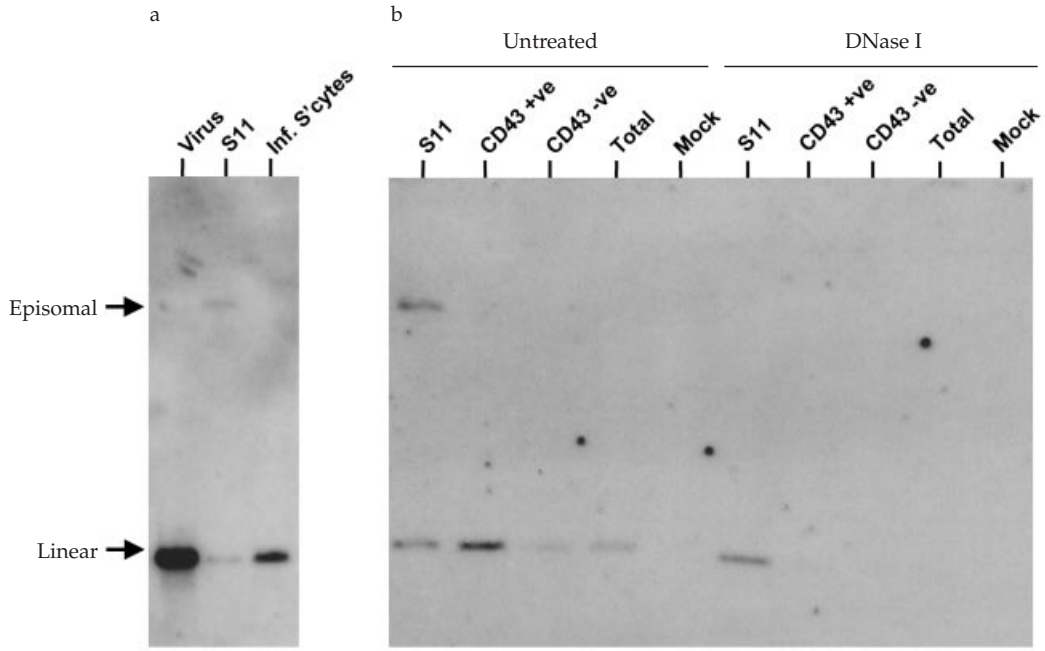

Fig. 5. The conformation of virus genome within cells (1×10^6 cells per lane) was determined by Gardella gel analysis. (*a*) Analysis of intact cells. Cells used were virus-infected BHK cells (Virus), S11 tumour cells or *in vitro*-infected splenocytes (Inf. S'cytes). (*b*) Analysis of nuclei. Nuclei were loaded either untreated or after DNase I treatment as indicated above the lanes. Cells used were S11, infected total splenocytes (Total), mock-infected total splenocytes (Mock), infected CD43-negative (resting) B cells (CD43 −ve) and infected CD43-positive cells (CD43 +ve). The relative positions of episomal and linear genomes are shown by arrows on the left.

Discussion

Studies on virus infection in cultured cells have proven to be useful models for elucidating patterns of gene expression for members of the herpesvirus family including alpha- and betaherpesviruses as well as gammaherpesviruses such as EBV. While much useful information can be obtained from these systems, it is clear that they have limitations. The studies on MHV-68 infection described here show that there are major differences between infection of isolated B cells *in vitro* and the events that occur *in vivo*. While the virus can infect lymphocytes *in vitro* and undergo limited transcription as well as alter the cell phenotype, the *in vitro* infection appears to be an abortive infection that results neither in the productive replication of the virus nor in the establishment of latent infection with growth transformation of B cells. Similar conclusions have been reached recently by Stevenson & Doherty (1999), who have shown that phenotypic changes *in vitro* are independent of T cells, whereas T cells are required *in vivo* for changes in B cell phenotype and immunoglobulin synthesis. Our results show that a major difference between infection *in vitro* and *in vivo* is the absence of significant amounts of circular virus genomes in B cells infected *in vitro*. This observation is likely to underlie many of the differences between infection *in vitro* and *in vivo*.

The prevailing model for herpesvirus infection proposes that the input viral DNA circularizes soon after infection (Roizman & Sears, 1996). This is supported by studies with a BHK cell temperature-sensitive mutant cell line, tsBN2, that undergoes premature condensation of chromatin at the non-permissive temperature. Failure to circularize in these cells shows that, at least in herpes simplex virus type 1, this process is dependent on a cellular gene product (Umene & Nishimoto, 1996). In addition, evidence from latent infections of the gammaherpesviruses EBV, HVS and HHV-8 indicates that the viral DNA is maintained and replicated as an episome (Decker *et al.*, 1996*b*; Gardella *et al.*, 1984). Our studies indicate a failure of the MHV-68 genome to circularize to any great extent in splenocytes infected *in vitro*. While we cannot exclude the possibility that circularization occurs in a limited number of cells, below the limit of detection in our assay, it is clear that the majority of the viral DNA is present in a linear, uncoated form. MHV-68 DNA circularizes *in vivo* (Stewart *et al.*, 1998) and in myeloma cell lines such as the NS0 cell line infected *in vitro* (Sunil-Chandra *et al.*, 1993). Our studies support the hypothesis that, during infection *in vivo*, specific activation of infected lymphocytes is required to induce synthesis of a cellular gene that allows circularization of the virus genome and allows infection to proceed. Thus, while the virus is capable of infecting and initiating limited transcription in all B cells *in vitro*, this infection is clearly abortive, since the expression of known lytic and latency-associated genes is absent. The rapid expansion of latently infected B cells seen in the spleen after MHV-68 infection is clearly dependent upon

$CD4^+$ T cell 'help' (Ehtisham *et al.*, 1993; Usherwood *et al.*, 1996 *a*). It is therefore likely that, *in vivo*, infection of B cells only results in latent or productive infection after the delivery of specific activation signals to the B cell. Such a signal(s) may be delivered by MHV-68-specific T cells. An additional restraint may be that infection can only proceed in a specific B cell; for example, a B cell with a defined cell phenotype or, indeed, an MHV-68-specific B cell.

It is clear from the work described here that, under a wide range of conditions that might activate and support cellular proliferation, MHV-68 does not readily transform growth of murine lymphocytes *in vitro*. We cannot, however, discount the association of MHV-68 with cellular transformation *in vivo* or rule out the possibility that, given the required conditions, MHV-68 is capable of growth transformation of murine lymphocytes *in vitro*. Circularization of the MHV-68 genome, which is a prerequisite for productive and persistent infections, was not detectable in virus-infected unstimulated lymphocyte cultures or in infected resting B cells, indicating that these conditions did not mimic *in vivo* events. While we have not examined the form of the genome under a wide range of conditions, the experiments suggest that it may be necessary to investigate culture conditions that support circularization of the virus genome before conclusions on the growth-transforming ability of MHV-68 can be drawn.

This work was funded by the Medical Research Council (UK) and The Royal Society. J.P.S. is a Royal Society University Research Fellow. The authors wish to thank Dr Chris Preston, MRC Virology Unit, Glasgow, for helpful discussions.

References

Alfieri, C., Birkenbach, M. & Kieff, E. (1991). Early events in Epstein–Barr virus infection of human B lymphocytes. *Virology* **181**, 595–608.

Biesinger, B., Muller-Fleckenstein, I., Simmer, B., Lang, G., Wittmann, S., Platzer, E., Desrosiers, R. C. & Fleckenstein, B. (1992). Stable growth transformation of human T lymphocytes by herpesvirus saimiri. *Proceedings of the National Academy of Sciences, USA* **89**, 3116–3119.

Blaskovic, D., Stancekova, M., Svobodova, J. & Mistrikova, J. (1980). Isolation of five strains of herpesviruses from two species of free living small rodents. *Acta Virologica* **24**, 468.

Bowden, R. J., Simas, J. P., Davis, A. J. & Efstathiou, S. (1997). Murine gammaherpesvirus 68 encodes tRNA-like sequences which are expressed during latency. *Journal of General Virology* **78**, 1675–1687.

Clarke, A. R. (1995). Murine models of neoplasia: functional analysis of the tumour suppressor genes Rb-1 and p53. *Cancer and Metastasis Reviews* **14**, 125–148.

Clarke, A. R., Maandag, E. R., van Roon, M., van der Lugt, N. M. T., van der Valk, M., Hooper, M. L., Berns, A. & te Riele, H. (1992). Requirement for a functional Rb-1 gene in murine development. *Nature* **359**, 328–330.

Clarke, A. R., Purdie, C. A., Harrison, D. J., Morris, R. G., Bird, C. C., Hooper, M. L. & Wyllie, A. H. (1993). Thymocyte apoptosis induced by p53-dependent and independent pathways. *Nature* **362**, 849–852.

Decker, L. L., Klaman, L. D. & Thorley-Lawson, D. A. (1996*a*). Detection of the latent form of Epstein–Barr virus DNA in the peripheral blood of healthy individuals. *Journal of Virology* **70**, 3286–3289.

Decker, L. L., Shankar, P., Khan, G., Freeman, R. B., Dezube, B. J., Lieberman, J. & Thorley-Lawson, D. A. (1996*b*). The Kaposi sarcoma-associated herpesvirus (KSHV) is present as an intact latent genome in KS tissue but replicates in the peripheral blood mononuclear cells of KS patients. *Journal of Experimental Medicine* **184**, 283–288.

Efstathiou, S., Ho, Y. M. & Minson, A. C. (1990). Cloning and molecular characterization of the murine herpesvirus 68 genome. *Journal of General Virology* **71**, 1355–1364.

Ehtisham, S., Sunil-Chandra, N. P. & Nash, A. A. (1993). Pathogenesis of murine gammaherpesvirus infection in mice deficient in CD4 and CD8 T cells. *Journal of Virology* **67**, 5247–5252.

Ernberg, I., Falk, K. & Hansson, M. (1987). Progenitor and pre-B lymphocytes transformed by Epstein–Barr virus. *International Journal of Cancer* **39**, 190–197.

Fickenscher, H. & Fleckenstein, B. (1998). Growth transformation of human T cells. *Methods in Medical Microbiology* **25**, 573–603.

Flore, O., Rafii, S., Ely, S., O'Leary, J. J., Hyjek, E. M. & Cesarman, E. (1998). Transformation of primary human endothelial cells by Kaposi's sarcoma-associated herpesvirus. *Nature* **394**, 588–592.

Gardella, T., Medveczky, P., Sairenji, T. & Mulder, C. (1984). Detection of circular and linear herpesvirus DNA molecules in mammalian cells by gel electrophoresis. *Journal of Virology* **50**, 248–254.

Gordon, J., Walker, L., Guy, G., Brown, G., Rowe, M. & Rickinson, A. (1986). Control of human B-lymphocyte replication. II. Transforming Epstein–Barr virus exploits three distinct viral signals to undermine three separate control points in B-cell growth. *Immunology* **58**, 591–595.

Hurley, E. A. & Thorley-Lawson, D. A. (1988). B cell activation and the establishment of Epstein–Barr virus latency. *Journal of Experimental Medicine* **168**, 2059–2075.

Husain, S. M., Usherwood, E. J., Dyson, H., Coleclough, C., Cappola, M., Woodland, D. L., Blackman, M., Stewart, J. P. & Sample, J. T. (1999). Murine gammaherpesvirus M2 gene is latency associated and a target for $CD8^+$ T lymphocytes. *Proceedings of the National Academy of Sciences, USA* **96**, 7508–7513.

Jamieson, D. R. S., Robinson, L. H., Daksis, J. I., Nicholl, M. J. & Preston, C. M. (1995). Quiescent viral genomes in human fibroblasts after infection with herpes simplex virus type 1 Vmw65 mutants. *Journal of General Virology* **76**, 1417–1431.

Nash, A. A., Usherwood, E. J. & Stewart, J. P. (1996). Immunological features of murine gammaherpesvirus infection. *Seminars in Virology* **7**, 125–130.

Raab-Traub, N. (1996). Pathogenesis of Epstein–Barr virus and its associated malignancies. *Seminars in Virology* **7**, 315–323.

Rickinson, A. B. & Kieff, E. (1996). Epstein–Barr virus. In *Field's Virology*, 3rd edn, pp. 2397–2446. Edited by B. N. Fields, D. M. Knipe & P. M. Howley. Philadelphia: Lippincott–Raven.

Roizman, B. & Sears, A. E. (1996). Herpes simplex viruses and their replication. In *Field's Virology*, 3rd edn, pp. 2231–2295. Edited by B. N. Fields, D. M. Knipe & P. M. Howley. Philadelphia: Lippincott–Raven.

Roizmann, B., Desrosiers, R. C., Fleckenstein, B., Lopez, C., Minson, A. C. & Studdert, M. J. (1992). The family *Herpesviridae*: an update. The Herpesvirus Study Group of the International Committee on Taxonomy of Viruses. *Archives of Virology* **123**, 425–449.

Schirm, S., Muller, I., Desrosiers, R. C. & Fleckenstein, B. (1984). Herpesvirus saimiri DNA in a lymphoid cell line established by in vitro transformation. *Journal of Virology* 49, 938–946.

Schulz, T. F. (1998). Kaposi's sarcoma-associated herpesvirus (human herpesvirus-8). *Journal of General Virology* **79**, 1573–1591.

Sinclair, A. J. & Farrell, P. J. (1995). Host cell requirements for efficient infection of quiescent primary B lymphocytes by Epstein–Barr virus. *Journal of Virology* **69**, 5461–5468.

Stevenson, P. G. & Doherty, P. C. (1999). Non-antigen-specific B-cell activation following murine gammaherpesvirus infection is CD4 independent in vitro but CD4 dependent in vivo. *Journal of Virology* **73**, 1075–1079.

Stewart, J. P., Usherwood, E. J., Ross, A., Dyson, H. & Nash, T. (1998). Lung epithelial cells are a major site of murine gammaherpesvirus persistence. *Journal of Experimental Medicine* **187**, 1941–1951.

Stuart, A. D., Stewart, J. P., Arrand, J. R. & Mackett, M. (1995). The Epstein–Barr virus encoded cytokine viral interleukin-10 enhances transformation of human B lymphocytes. *Oncogene* **11**, 1711–1719.

Sunil-Chandra, N. P., Efstathiou, S., Arno, J. & Nash, A. A. (1992*a*). Virological and pathological features of mice infected with murine gammaherpesvirus 68. *Journal of General Virology* **73**, 2347–2356.

Sunil-Chandra, N. P., Efstathiou, S. & Nash, A. A. (1992*b*). Murine gammaherpesvirus 68 establishes a latent infection in mouse B lymphocytes *in vivo*. *Journal of General Virology* **73**, 3275–3279.

Sunil-Chandra, N. P., Efstathiou, S. & Nash, A. A. (1993). Interactions of murine gammaherpesvirus 68 with B and T cell lines. *Virology* **193**, 825–833.

Sunil-Chandra, N. P., Arno, J., Fazakerley, J. & Nash, A. A. (1994). Lymphoproliferative disease in mice infected with murine gammaherpesvirus 68. *American Journal of Pathology* **145**, 818–826.

Tripp, R. A., Hamilton-Easton, A. M., Cardin, R. D., Nguyen, P., Behm, F. G., Woodland, D. L., Doherty, P. C. & Blackman, M. A. (1997). Pathogenesis of an infectious mononucleosis-like disease induced by a murine gamma-herpesvirus: role for a viral superantigen? *Journal of Experimental Medicine* **185**, 1641–1650.

Umene, K. & Nishimoto, T. (1996). Replication of herpes simplex virus type 1 DNA is inhibited in a temperature-sensitive mutant of BHK-21 cells lacking RCC1 (regulator of chromosome condensation) and virus DNA remains linear. *Journal of General Virology* **77**, 2261–2270.

Usherwood, E. J., Ross, A. J., Allen, D. J. & Nash, A. A. (1996*a*). Murine gammaherpesvirus-induced splenomegaly: a critical role for CD4 T cells. *Journal of General Virology* **77**, 627–630.

Usherwood, E. J., Stewart, J. P. & Nash, A. A. (1996*b*). Characterization of tumor cell lines derived from murine gammaherpesvirus-68-infected mice. *Journal of Virology* **70**, 6516–6518.

Weck, K. E., Kim, S. S., Virgin, H. W., IV & Speck, S. H. (1999). Macrophages are the major reservoir of latent murine gammaherpesvirus 68 in peritoneal cells. *Journal of Virology* **73**, 3273–3283.

Received 6 April 1999; Accepted 17 June 1999