Absence of splenic latency in murine gammaherpesvirus 68-infected B cell-deficient mice

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Murine gammaherpesvirus 68 (MHV-68) is a natural pathogen of mice which causes an acute lung infection and establishes a latent infection in B lymphocytes. In this paper we describe the infection in transgenic B cell-deficient (μMT) mice, to determine whether a latent infection can be established in a mouse lacking circulating B lymphocytes. Little difference was observed in the acute lung infection, although there was a slight delay in virus clearance in the μMT mice. This indicates that antiviral antibody is of little importance in the resolution of the lung infection. Neither free nor latent virus could be detected in the spleen in the μMT mice. In addition, these mice did not develop MHV-68-induced splenomegaly. These data suggest that within the lymphoid compartment B lymphocytes are the sole reservoir for MHV-68 infection in vivo, confirming earlier work which identified B cells as the site of latent infection based on cell fractionation studies. In addition, our study shows that CD4-driven lymphocyte expansion leading to splenomegaly is dependent on the presence of MHV-68-infected B cells in the spleen. Although no free virus was detected (using conventional biological assays) in the lung after the resolution of the acute infection, MHV-68 genome was detected in the lungs of both control and μMT mice by PCR analysis. This suggests that cells in the lung may act as a reservoir of latent virus which is independent of the B lymphocyte infection.

Introduction

Murine gammaherpesvirus 68 (MHV-68) can be regarded as a useful model for gammaherpesvirus infection in a natural host species. MHV-68 was isolated from a free-living murid rodent (Blaskovic et al., 1980), and is classified as a gammaherpesvirus on the basis of limited sequence data, genome structure and biology (Efstathiou et al., 1990a, b). After intranasal inoculation, virus replication occurs in the lung for 7–10 days (Sunil-Chandra et al., 1992b) and is subsequently cleared. The infection then spreads to the spleen and infected mice develop a marked splenomegaly during the second week post-infection. A latent infection is established in the B lymphocyte compartment (Sunil-Chandra et al., 1992a, 1993), and at late times post-infection a proportion of mice develop lymphoproliferative disease (Sunil-Chandra et al., 1994). Characterization of latently infected cells grown from tumour explants (Usherwood et al., 1996b) or lines of B cell origin infected in vitro (Sunil-Chandra et al., 1993) have revealed a pattern of infection similar to that observed for other gammaherpesviruses such as herpesvirus saimiri and Epstein–Barr virus (EBV). MHV-68 persists indefinitely in the myeloma cell line NS0, without any cytopathic effect, but with the production of infectious virus. Production of virus was abolished by treatment with acyclovir, but large numbers of latently infected cells remained in the culture (Sunil-Chandra et al., 1993). Analysis of the viral DNA showed the genome was persisting in an episomal form during the latent infection.

Both cytotoxic CD8 T cells and ‘helper’ CD4 T cells play a role in control of the infection. Mice depleted of CD8 T cells were unable to clear the lung infection (Ehtisham et al., 1993), and recent evidence suggests CD8 T cells also have a role in the control of latently infected B cells in the spleen (Nash et al., 1996). CD4 T cells are of less importance in the lung – depleted animals have only a slight delay in the kinetics of virus clearance – but they are essential for the development of splenomegaly (Usherwood et al., 1996a). Cells from the spleen and lung-draining lymph nodes have been shown to produce high levels of IL-6 and IFN-γ and lower levels of IL-2 and IL-10 following MHV-68 infection (Sarawar et al., 1996). The role of antibody in this infection has not yet been established.
To further study the role of B lymphocytes and antiviral antibodies in the host response to MHV-68 we analysed the infection in mice deficient in B lymphocytes. In the absence of the principal target cell for latency it was possible to address whether the virus had an additional tropism for other cell types in the latent infection. The mice used in this study had a disruption in one of the membrane exons of the µ immunoglobulin chain (µMT/µMT mice; Kitamura et al., 1991). These mice possess B220+ pre-B cells in the bone marrow, but B cell development is arrested at this stage and there are no B220+ or IgM+ B cells in the spleen. Using these mice, we observed no infective centres or viral DNA in the spleen, suggesting they were unable to establish a latent infection in this organ. In addition, µMT mice failed to develop splenomegaly, although the ability to clear virus from the lung was only marginally impaired indicating that antibody is not required for resolution of the acute respiratory infection. However, virus DNA was detected by PCR in the lungs of both control and µMT mice at late time-points, suggesting that this organ is an alternative site of latency independent of B cell infection.

**Methods**

- **Virus.** Virus stocks were originally obtained from D. Blaskovic (Blaskovic et al., 1980) and clone G2.4 was isolated (Efstathiou et al., 1990a, b). Virus was propagated in BHK cells and working stocks stored at —70 °C.

- **Mice.** The mice used in this study were µMT/µMT mice (Kitamura et al., 1991) on a C57BL/6 background. They have a targeted lesion in the µ-immunoglobulin chain resulting in the failure to express IgM; consequently, B cell development is arrested at the pre-B cell stage. A colony was established from these mice in the Department of Veterinary Pathology, University of Edinburgh. C57BL/6 mice were obtained from Bantin and Kingman, UK. Male and female µMT and C57BL/6 mice were infected at 4—11 weeks of age intranasally with 4 x 10⁶ p.f.u. MHV-68 under halothane anaesthesia.

- **Assay for infectious virus and infective centres.** Virus plaque assays were performed on BHK cell monolayers as previously described (Sunil-Chandra et al., 1992b). An infective centre assay was utilized for detecting latently infected cells. Briefly, spleen cell suspensions were subjected to water lysis to lyse red blood cells, then co-cultivated with BHK cells for 5 days and numbers of cells which reactivated virus to give rise to a plaque were calculated (Sunil-Chandra et al., 1992b).

- **Lung co-cultivation.** Lungs were removed and placed into ice-cold medium; the larger airways were then removed, along with all lung-associated lymphoid and connective tissue. The tissue was cut into pieces of approximately 1—2 mm³, then added to a 60 mm Petri dish containing 5 ml of medium plus 10⁴ BHK cells. After 5 days incubation at 37 °C medium was removed and the BHK monolayer detached with a 1 ml rubber syringe plunger and pooled with lung pieces in a volume of approximately 1 ml. Samples were stored at —80 °C until required, then homogenized and titrated as for other lung samples (Sunil-Chandra et al., 1992b).

- **ELISA for anti-MHV-68 antibody.** Serum samples were prepared from blood extracted by cardiac puncture. Dynatech Immulon 4 ELISA plates were coated with rabbit hyperimmune anti-MHV-68 serum (Sunil-Chandra et al., 1992b) in carbonate–bicarbonate buffer at pH 9.8 overnight at 4 °C. Plates were washed five times in borate-buffered saline plus 0.001% Tween 20 (BBS–Tween) after incubation with each reagent. Blocking of non-specific binding was accomplished by incubating with 2% normal rabbit serum at 37 °C for 1 h. UV-irradiated MHV-68 was added to each well in BBS–Tween plus 1% normal rabbit serum at a concentration of approximately 10⁶ p.f.u./ml. Two-fold serial dilutions of mouse sera were prepared, in the range 1/20—1/80, added to the plates and incubated at 37 °C for 90 min. Normal mouse serum and mouse hyperimmune anti-MHV-68 serum acted as negative and positive controls respectively. Specific binding was detected with horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulins (Dako), incubated in the plates for 1 h at 37 °C. Plates were developed with o-phenylenediamine dihydrochloride (OPD) in citrate–phosphate buffer at pH 5.0, the reaction stopped after 15—20 min with 12.5% H₂SO₄ and absorbance quantified at 490 nm on a Dynatech MRS000 ELISA plate reader.

- **Cytofluorometry.** Staining for the lymphocyte surface markers CD4, CD8 and IgM(µ) was performed as previously described (Usherwood et al., 1996a). Briefly, aliquots of 5 x 10⁶ spleen cells were stained with primary antibodies for 20 min on ice, and washed three times in ice-cold PBS plus 1% BSA and 0.001% sodium azide. The cells were then stained with secondary FITC-conjugated antibodies or streptavidin–FITC for a further 20 min on ice and then washed and fixed in 1% formaldehyde in PBS. Samples were analysed with a Becton-Dickinson FACScan. Anti-CD8 and anti-CD4 antibodies were from hybridomas YTS 169 and YTS 191 respectively (Cobbold et al., 1984); biotin-conjugated anti-µ was purchased from Sera-lab.

- **PCR analysis.** All DNA extraction procedures and set-up of PCR reactions were physically separated from the analysis of PCR products and all procedures performed using aerosol-resistant pipette tips (Chudia, Bio-Rad) to eliminate false-positive results due to contamination of PCR reactions. High molecular mass DNA was extracted from spleen or lung biopsies using a QIAamp tissue kit (Qiagen) according to the manufacturer’s instructions. Accurate quantification of DNA concentration was performed using a DNA quant 200 fluorimeter (Hoefer). PCR amplification to detect MHV-68 DNA was performed on 200 ng of high molecular mass DNA using primers specific for the MHV-68 gp150 gene described by Stewart et al., (1996). Reactions were performed in a total of 100 µl using 1:5 units of Taq DNA polymerase (Boehringer) in buffer supplied by the manufacturer containing 1.5 mM-MgCl₂ for 40 cycles of 94 °C for 30 s, 57 °C for 1 min and 72 °C for 1 min. This reaction produced a 501 bp product on positive control DNA. Nested PCR was performed on 10 µl of product from the above reaction using the primers 5′ CAC CTC AGA ACC AAC T TC 3′ (sense) and 5′ GTA TCT GAT GTG TCA GCA G 3′ (antisense), which lie internal to the first set on the target DNA. Reactions were as for the first round except that they were performed for 25 cycles of 94 °C for 30 s, 49 °C for 1 min and 72 °C for 1 min. This reaction produced a 368 bp product on positive control DNA. All products were analysed by electrophoresis through gels containing 2% agarose and ethidium bromide.

The approximate sensitivity of the reactions was tested as follows. Serial dilutions of a known quantity of a plasmid containing the gp150 gene were made down to one copy per tube in the presence of MHV-68-negative high molecular mass spleen DNA. PCR reactions were then performed on each dilution in the presence of 200 ng of MHV-68-negative spleen DNA. The experiment was performed on three separate occasions with comparable results as follows. The first round of PCR (40
cycles) could detect 10^8 copies of virus DNA and the second (nested) round (65 cycles) 10 copies.

An alternative PCR amplification to detect MHV-68 DNA was used to confirm the results obtained with some DNA samples. This used primers known to be specific for the MHV-68 gene encoding the homologue of the EBV M trans-activator protein or BMLF1 (S. Pepper, J. P. Stewart, J. R. Arrand & M. Mackett, unpublished observations). The primers used were 5' ATG GCA CAG CAG ATG TTG GA 3' (sense) and 5' TAT CTC TAA GCA GAG GAG AG 3' (antisense) and the reactions performed as above for 40 cycles of 94 °C for 30 s, 53 °C for 1 min and 72 °C for 1 min.

PCR amplification to detect the single copy mouse genomic gene which encodes the perforin molecule was performed using primers and under conditions described by Kaegi et al. (1994).

Results

Antibody response to MHV-68

Groups of μMT and control C57BL/6 mice were infected by the intranasal route with 4 × 10^8 p.f.u. MHV-68. At various times post-infection sera from these mice were assayed for levels of antiviral antibodies using a capture ELISA. Results are shown in Fig. 1. In C57BL/6 mice the antibody titre rose rapidly starting on day 7 post-infection to reach a plateau between days 28–35. No antiviral antibody was detected in sera from infected μMT mice.

Respiratory infection in μMT mice

Lungs were removed from MHV-68-infected C57BL/6 and μMT mice at various times after infection and titred for the presence of infectious virus. As shown in Fig. 2, high titres of virus were detected in control mice at days 3 and 7, but by day 10 virus had been cleared from the lung and was not detectable at any subsequent time-point. However, in the μMT mice, although virus titres at days 3 and 7 were comparable to those in control mice, virus was also detected in 2/4 mice at day 14 post-infection. After day 14 no virus was detected in the lungs of μMT mice. This delay in virus clearance, though slight, was reproducible (data not shown). This indicates that although antiviral antibody may contribute to the clearance of the virus from the lung, it is not essential. The anti-MHV-68 antibody response is only starting to rise by day 7 post-infection (Fig. 1), when virus clearance is beginning, suggesting the response is not rapid enough to have a marked effect on the lung virus titre during the acute infection.

Splenomegaly in μMT mice

Splenomegaly is usually observed in MHV-68-infected mice, beginning during the second week of infection and resolves 2–3 weeks later (Sunil-Chandra et al., 1992b; Usherwood et al., 1996a). MHV-68-infected C57BL/6 mice developed splenomegaly (Fig. 3), showing a twofold increase in the number of spleen cells at day 10 post-infection. No statistically significant splenomegaly was observed in infected μMT mice.

Lymphocyte populations present in splenomegaly

Previous reports from this laboratory have shown that during splenomegaly there is an increase in the number of B lymphocytes, CD4 lymphocytes and particularly CD8 lymphocytes (Usherwood et al., 1996a). Although there was no overall increase in the number of spleen cells observed in MHV-68-infected μMT mice, it was possible that there was an expansion in some lymphocyte subsets which was counter-balanced by a decrease in other populations. To investigate this, splenocytes from infected C57BL/6 and μMT were stained with anti-CD4, anti-CD8 and anti-IgM and the proportion of each lymphocyte subset was measured by flow cytometry. An expansion in the B cell and both T cell populations was observed in infected C57BL/6 mice (Fig. 4). The numbers of CD4 and CD8 T cells did not change following MHV-68 infection of μMT mice, indicating no expansion in the T cell population. No IgM-positive cells were detectable in μMT mice (Fig. 4).

Establishment of latency in μMT mice

The number of latently infected splenic lymphocytes was estimated by infective centre assay, and is shown in Table 1. Infective centre levels in control C57BL/6 mice rose to high levels at days 10–14 post-infection, then fell to approximately 100 infective centres per spleen. We consistently failed to detect infective centres at any time during infection of μMT mice (Table 1), indicating that there were no latently infected cells capable of reactivating virus in vitro. To address the possibility of a latent infection in a cell type that did not reactivate virus in vitro, DNA from spleen cells was prepared and subjected to nested PCR analysis. Viral DNA was detectable in samples from C57BL/6 mice by using nested PCR (65 cycles) at 35 days post-infection; however, none was detected in μMT samples (Fig. 5). Similar data were obtained from samples taken at 28 days post-infection (data not shown). To confirm that the DNA was of sufficient quality to be amplified, samples which were negative for MHV-68 by PCR were re-analysed and all found to be positive when primers specific for the single copy mouse genomic gene which encodes the perforin molecule were used. The limit of sensitivity of the nested PCR for MHV-68 was approximately 10 genome copies per sample, so the absence of detectable virus DNA in μMT spleens suggests there was no latent virus associated with lymphoid tissue.

Infectious virus was not detectable in the lungs of either group of mice after 14 days post-infection. However, virus DNA was detected in all mice tested at 35 days post-infection by a single round of PCR (40 cycles) using primers specific for
Fig. 1. Antibody response to MHV-68 in µMT and control mice. Serum was prepared from infected C57BL/6 (□) and µMT (○) mice at various times after infection and analysed for the presence of antiviral antibody using an EUSA system, as described in Methods. Results shown were obtained using serum diluted 1/20. Points represent mean readings of four mice per time-point; error bars show one standard deviation.

Fig. 2. Lung virus titres in infected µMT and control mice. Lungs were removed, homogenized and assayed for the presence of infectious virus. Each point represents the lung titre from a single mouse; the dotted line shows the limit of detection of the assay (10 p.f.u. per sample). □, C57BL/6 mice; ○, µMT mice.

Fig. 3. MHV-68-induced splenomegaly in µMT and control mice. Results shown are the mean and standard deviation of the number of spleen cell from groups of four mice at various times post-infection. □, C57BL/6 means; ○, µMT means. Typical values for uninfected C57BL/6 and µMT mice are 10⁸ and 3 x 10⁷ respectively.

The level of virus was detected sporadically in the equivalent µMT samples (data not shown). This suggests that lung-associated virus was present in both C57BL/6 and transgenic animals in a latent form but was not reliably reactivatable using the culture conditions described.

Discussion

The lack of any latently infected cells in B lymphocyte-deficient mice indicates that, at least in lymphoid tissue, all of the cells capable of sustaining a latent infection reside in the B lymphocyte compartment. This concurs with previous data from this laboratory showing that in cell fractionation studies the majority of infective centres arise from the B cell fraction (Sunil-Chandra et al., 1992a). In vitro infection of established lymphoid cell lines also showed that cells of the B lineage (NS0) but not the T lineage (BW5147) could support a latent infection (Sunil-Chandra et al., 1993).

EBV also resides in B lymphocytes during latent infection. It is thought that after initial infection of the B cell compartment there is a period of rapid expansion in the infected B cell pool during which the full panel of latency-associated genes are expressed. A switch may then occur resulting in down-regulation of all latent proteins with the exception of EBNA-1 and LMP-2 (Qu & Rowe, 1992; Tierney et al., 1994; Chen et al., 1995). The infected B cells then exit the cell cycle and revert to the resting phenotype (Miyashita et al., 1995). Thus, the latent reservoir is in resting B cells, which may periodically reactivate to expand the latent cell pool or re-seed epithelial tissue where a productive infection can occur. Although nothing is yet known about genes expressed during MHV-68 latency, a similar pattern of events can be envisaged, where an expansion...
Table 1. Infective centres in μMT and C57BL/6 mice infected with MHV-68

<table>
<thead>
<tr>
<th>Time post-infection (days)</th>
<th>No. of infective centres per spleen in C57BL/6 mice</th>
<th>No. of infective centres per spleen in μMT mice</th>
</tr>
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<tbody>
<tr>
<td>7</td>
<td>43 ± 38</td>
<td>&lt; 10</td>
</tr>
<tr>
<td>10</td>
<td>&gt; 5000</td>
<td>&lt; 10</td>
</tr>
<tr>
<td>14</td>
<td>&gt; 5000</td>
<td>&lt; 10</td>
</tr>
<tr>
<td>17</td>
<td>2310 ± 1100</td>
<td>&lt; 10</td>
</tr>
<tr>
<td>21</td>
<td>885 ± 558</td>
<td>&lt; 10</td>
</tr>
<tr>
<td>28</td>
<td>406 ± 216</td>
<td>&lt; 10</td>
</tr>
<tr>
<td>35</td>
<td>161 ± 78</td>
<td>&lt; 10</td>
</tr>
</tbody>
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Fig. 4. Lymphocyte populations in spleens of μMT and control mice. Spleen cell suspensions were stained with anti-CD4, anti-CD8 and anti-IgM antibodies, followed by FITC-labelled secondary reagent and analysed using a Becton-Dickinson FACScan. The size of each lymphocyte population was calculated from the percentage of cells staining for the appropriate antigen and the total spleen cell number. Each point represents a mean value from four mice; error bars show one standard deviation. □, C57BL/6 means; ◆, μMT means. An indication of the numbers in each population in uninfected C57BL/6 mice is shown by dotted lines.

Antiviral antibody appears to be of less importance than cell-mediated immunity in the response to EBV. Patients with infectious mononucleosis (IM) develop IgM and IgG responses to viral capsid antigens (VCA) and early antigens (EA), although neutralizing anti-gp350 antibodies are slow to develop (Henle & Henle, 1979). As infectious mononucleosis resolves the antibody profile becomes the same as that seen in virus carriers, namely anti-VCA, anti-EBNA-I, anti-gp350 neutralizing antibody. It is unlikely that antiviral antibody is significant once a latent infection has been established, although elevated titres are a useful diagnostic indicator of the onset of EBV-related malignancies (Henle & Henle, 1976, 1979). In the acute infection, neutralizing antibody may limit the spread of free virus in the blood and non-neutralizing antibody may contribute to antibody-dependent cell-mediated cytotoxicity. The lack of antibody in MHV-68-infected μMT mice resulted in only a slight delay in clearance of virus from the lung, suggesting that antibody is not necessary for clearance from this site, but may accelerate the process. Previous work from this laboratory showed that CD8-depleted mice cannot clear the lung infection, although CD4 depletion only delays the clearance (Ehtisham et al., 1993). As both CD4 depletion and a lack of antibody result in a similar delay in clearance, it is possible that CD4 cell removal deprives B cells of immunological help, resulting in a weaker antibody response. Overall, it appears that antiviral CD8 T cells are more important than antibody in the acute respiratory infection with MHV-68, making it likely that the same is true for EBV.

Another interesting feature of the infection in μMT mice was the lack of splenomegaly. CD4 T cells are absolutely required for the development of splenomegaly (Usherwood et al., 1996a), and the present data indicate that infected B cells are also required. As described above, MHV-68 replicates in the lungs of μMT mice for up to 14 days, during which time CD4 T cell priming would be expected to occur in the draining mediastinal lymph node (Allan et al., 1990; Xie et al., 1995). However, this is clearly not sufficient to drive the cellular

of latently infected cells occurs during splenomegaly (Sunil-Chandra et al., 1992b). Numbers of infective centres then fall to a relatively stable level of approximately one infective centre per 10⁶ spleen cells.

There are several possible reasons for this. The majority of infected lymphocytes may be killed by the immune system (probably antiviral CTLs), leaving a small pool which are able to evade immune-mediated clearance. Apoptosis of infected cells may also contribute to a fall in infective centre levels. A second theory involves a change in the form of latency to a form where infected cells are not seen by the antiviral immune response. With this model, the fall in infective centres may be an illusion due to the failure of the assay used to detect latently infected cells. Our results indicate that if this is the case the non-reactivatable cells are also of the B lymphocyte lineage, as no virus DNA was detected by sensitive nested PCR in spleen tissue lacking mature B cells.
proliferation necessary to produce splenomegaly. The likely reason for this is the lack of infected B cells presenting antigen in the spleen which results in no significant clonal expansion at this site, even if CD4 T cells are primed in the lung.

There is conflicting evidence concerning the role of B cells in CD4 T cell priming (Constant et al., 1995; Epstein et al., 1995), and B cells may be necessary for CD4 responses against some antigens but not others. These experiments were performed with soluble antigens which may prime in a very different way to a B cell-associated intracellular virus antigen. The issue of CD4 T cell priming in MHV-68-infected μMT mice is currently under investigation, but it is clear that complex T–B interactions are involved in the priming and regulation of the immune response to this virus.

The finding that lung tissue harbours MHV-68 long after the acute infection is of great interest. This phenomenon occurs in the absence of B lymphocytes, and our data suggest that it may represent a latent infection rather than a low-level chronic infection, although further work is required to confirm this. It has been suggested that EBV may replicate solely in B lymphocytes, and spread when the B cells enter a lytic phase of replication when passing through the nasopharynx. According to this model infection of epithelial tissue is a secondary event to persistence in B cells. Other workers have reported the detection of EBV in cells exfoliated from the lung (Lung et al., 1985) and patients with the chronic lung disease cryptogenic fibrosing alveolitis (Egan et al., 1995), which suggests both that the lung may be a major reservoir for EBV and that its presence may be sometimes associated with lung pathology. Further characterization of the long-term lung infection in MHV-68 infected mice should help to shed light on the ability of gammaherpesviruses to establish a latent infection in lung tissue.

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References


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