Infection of intestinal epithelial cells and development of systemic disease following gastric instillation of murine gammaherpesvirus-68

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Murine gammaherpesvirus-68 (γHV-68) induces a lymphocytosis in mice and establishes a latent infection of B lymphocytes following intranasal administration in anaesthetized animals. Because γHV-68 is a gammaherpesvirus, it has been used as a model to understand the pathogenesis of Epstein–Barr virus (EBV) and human herpesvirus-8 (HHV-8) infections. In this study, we investigated the unlikely possibility that γHV-68 could survive the harsh gastrointestinal environment to efficiently infect intestinal epithelial cells, and then disseminate from mucosal sites to cause systemic disease. Surprisingly, oral administration, or gastric instillation which by-passed the oral cavity, readily caused a systemic lymphocytosis and established a latent infection in splenic leukocytes. The finding that γHV-68 could readily infect adult mice following gastric instillation strongly suggested that intestinal epithelial cells could be productively infected. Unlike the more routinely used method of intranasal inoculation, γHV-68 given intragastrically resulted in lytic virus, viral RNA and viral DNA being present in isolated intestinal epithelial cells. Furthermore, γHV-68 RNA and DNA, but not latent virus, could be detected in epithelial cells as long as 30 days post-infection, suggesting that some of these cells might be persistently infected. Taken together, these studies demonstrate that γHV-68 can survive passage through the gastrointestinal tract and infect intestinal epithelial cells. Following infection of gut epithelial cells, γHV-68 can disseminate from mucosal sites to induce a systemic lymphocytosis which is similar to the disease induced following intranasal inoculation.

Introduction

Murine gammaherpesvirus-68 (γHV-68) (Blaskovic et al., 1984) has recently been suggested as a model system to investigate the pathophysiology following human infection with Epstein–Barr virus (EBV) or Kaposi’s sarcoma-associated herpesvirus/human herpesvirus-8 (KSHV/HHV-8) (Doherty et al., 1997; Speck & Virgin, 1999; Virgin & Speck, 1999). Genetic analyses show that γHV-68 is a type 2 gammaherpesvirus (Efstathiou et al., 1990a, b) which induces a non-fatal mononucleosis-like disease in immune competent rodents (Sunil-Chandra et al., 1992a; Doherty et al., 1997). After instillation of the virus into the nasal cavity of anaesthetized mice, there is a productive infection of lung epithelial cells, followed by dissemination of the virus to peripheral organs (Sunil-Chandra et al., 1992a). B lymphocytes (Cardin et al., 1996; Sunil-Chandra et al., 1992b; Usherwood et al., 1996b) and possibly macrophages (Weck et al., 1999) become latently infected soon after inoculation followed by a marked splenomegaly (Cardin et al., 1996; Sunil-Chandra et al., 1992a; Usherwood et al., 1996a) due to expansion of T and B lymphocyte populations (Cardin et al., 1996; Tripp et al., 1997). Because of these similarities with gammaherpesvirus infections in humans, γHV-68 infection represents the first murine model system that is of use in helping to understand the host–pathogen interaction during infection with a leukotrophic gammaherpesvirus (Doherty et al., 1997; Nash & Sunil-Chandra, 1994; Simas & Efstathiou, 1998).

Investigations using the γHV-68 murine model have produced some surprising findings. For example, while B lymphocytes (Sunil-Chandra et al., 1992b; Usherwood et al., 1996b), and possibly macrophages (Weck et al., 1999), are...
considered the major cellular compartments for splenic latency, lung epithelial cells (Stewart et al., 1998) may also represent cells capable of serving as virus reservoirs. Other unexplained findings included the observation that despite being an intracellular pathogen, the production of IL-6 (Sarawar et al., 1996) or interferon-γ (Sarawar et al., 1997) during the initial/acute phase of infection seems to provide limited benefit to the host. Furthermore, while a viral superantigen has been hypothesized to contribute to the selective expansion of the Vβ4+ CD8+ T lymphocyte population (Tripp et al., 1997), there are most likely other, as yet unidentified, factors which augment this selective expansion (Doherty et al., 1997). Clearly, investigations of γHV-68-infected mice have contributed significantly to our understanding of gamma-herpesvirus infections, and have defined questions for future investigations (Doherty et al., 1997; Nash & Sunil-Chandra, 1994; Simas & Estathiou, 1998).

EBV initially infects epithelial cells of the nasopharynx, and this conclusion has been reached as a result of several lines of investigation. Scrapings of epithelial cells from acutely infected patients have demonstrated the presence of virus or viral genomes (Lemon et al., 1977; Sibbey et al., 1984). Furthermore, epithelial cells derived from the nasopharynx have been shown to be infectable following exposure to EBV in vitro (Shapiro & Volsky, 1983). The fact that cervical lymph nodes are often enlarged during mononucleosis also points to the nasopharynx as a site of infection. In addition, the high prevalence of nasopharyngeal carcinomas which are positive for EBV genomes or replicative virus has suggested that transformation can follow infection with this virus (Glaser et al., 1976; Kaschka-Dierich et al., 1976; Klein et al., 1974; Nonoyama & Pagano, 1973; Wolf et al., 1973).

Interestingly, there is also a strong association of EBV with gastric carcinomas. EBV genomes or replicative virus (Gulley et al., 1996; Imai et al., 1994; Seloves et al., 1996; Tokunaga et al., 1993) have been found in a significant percentage of gastric carcinomas. There have been some recent studies suggesting that gastric or intestinal epithelial cells can be directly infected by EBV (Tajima et al., 1998; Takasaka et al., 1998; Yanai et al., 1997). However, gastric or intestinal epithelial cells have not been considered as likely targets for EBV infection since the virus would have to survive the harsh environment of the gastrointestinal tract to infect these cell populations. If such cells were important targets for EBV infection, this finding would have important implications for carcinomas of the gastrointestinal tract.

In the present study we demonstrate that γHV-68 readily induces splenic leukocytosis and a latent infection of splenic leukocytes following oral or gastric inoculation. This result was especially surprising since gastric instillation of the virus bypassed the oral cavity. Furthermore, intestinal epithelial cells were found to be lytically infected by γHV-68 and to maintain expression of viral RNA and DNA even 30 days post-infection. Together these results demonstrate that γHV-68 can survive passage through the gastrointestinal tract to infect epithelial cells, followed by dissemination of the initial infection into the periphery.

**Methods**

- **Virus isolation.** γHV-68 was kindly provided by Tony Nash (University of Edinburgh, UK) and Peter Doherty (St Jude’s Hospital, Memphis, TN, USA). Virus stock was prepared by infecting BHK-21 cells (ATCC CCL 10) with γHV-68 at a low m.o.i. (0.1 p.f.u. per cell). After 24 h the cells were removed with trypsin and centrifuged to form a pellet. The pellet was pulse sonicated (Vibra Cell) at an output of 5 W to release intact virions. The sonicated material was centrifuged to remove unwanted cellular debris, and the supernatant, containing the virus, was split into aliquots and stored at −80 °C. Virus was quantified by making threefold serial dilutions on NIH-3T3 cell (ATCC CRL 1658) monolayers. The number of p.f.u. was determined for each viral preparation as described below. NIH-3T3 cells were maintained in RPMI 1640 (Gibco BRL) with 10% foetal calf serum (FCS) at 37 °C and 5% CO₂.

- **Intransal, oral and gastric inoculations with γHV-68.** BALB/c mice (Charles Rivers, Wilmington, MA, USA) were given food and water ad libitum, and were housed in isolation cages throughout the experimental period. Intransal inoculations with γHV-68 were performed as previously described (Cardin et al., 1991; Sunil-Chandra et al., 1992a). Briefly, mice were anaesthetized and allowed to aspirate, via the nasal passages, 20 µl of inoculum containing the indicated p.f.u. of γHV-68. Mice were allowed to recover from the anaesthesia prior to returning to their cages.

- **For oral inoculations, mice were restrained, but not anaesthetized. Dilutions of γHV-68 containing the indicated p.f.u. were diluted in a total of 50 µl of RPMI 1640. Mice readily consumed droplets of fluid from a pipette tip while being held, and the time required for administration was less than 1 min.**

- **For intragastric inoculations, mice were restrained, but not anaesthetized. Dilutions of γHV-68 containing the indicated p.f.u. were diluted in 500 µl of RPMI 1640 or saline and instilled directly into the stomach using a feeding tube connected to a 1 ml syringe. The feeding tube was cleaned before and after each instillation, and intragastric fluid was not aspirated during this procedure. It should be noted that these animals received no special treatment (e.g. neutralization of stomach acid, or withholding of food) prior to instillation.**

- **To assure that gastric intubation completely bypassed the oral cavity, a ‘mock’ intragastric intubation was also performed. A feeding tube connected to a 1 ml syringe containing 60,000 p.f.u. of virus per 500 µl was inserted gastrically into groups of mice and removed without instilling any fluid. These mice were then used as negative controls to demonstrate that insertion and removal of the feeding tube did not result in infection.**

- **Isolation of splenic leukocytes.** At the indicated times post-infection, spleens were removed and weighed. Single cell suspensions were made by pressing tissue through a 30 gauge wire-mesh screen, followed by centrifugation of cells on Hypaque-Ficoll gradients as previously described (Pascual et al., 1991). Mononuclear leukocytes were recovered from the interface, and counted.

- **Isolation of lung or intestinal epithelial cells.** Intestinal epithelial cells were isolated as previously described (Yamamoto et al., 1993). Briefly, following removal of the small intestines, 1 cm segments were placed in RPMI 1640 containing 2% FCS and gentamicin for 30 min
with gentle agitation. Intestinal segments were then placed in fresh medium and vigorously agitated for 15 s. This process was repeated, and the resulting supernatants were passed over nylon wool columns to remove debris. Cells were then separated on discontinuous Percoll gradients (Pharmacia), and epithelial cells isolated from the 30% interface.

Lung epithelial cells were isolated as previously described (Stewart et al., 1998). Lung tissue was diced into 3 mm$^3$ pieces and placed in RPMI 1640 containing 2% FCS and gentamicin for 10 min with gentle agitation. Tissue fragments were removed from the wash medium and incubated in RPMI 1640 containing gentamicin and 200 U/ml collagenase D (Boehringer Mannheim) for 60 min at 37 °C. Tissue was subsequently dissociated by passage through a 30 gauge wire mesh screen, and cells were then washed in medium containing 2% FCS.

Purity of the isolated epithelial cells was assessed by immunofluorescent staining for cytokeratin using a fluorescein-conjugated monoclonal antibody (clone C-11, Sigma). Essentially all of the isolated epithelial cells stained positive for cytokeratin, indicating that these cells were of epithelial cell origin (Benya et al., 1991; Chandler et al., 1991). Furthermore, to demonstrate the absence of contaminating B lymphocytes, a PCR to detect rearranged D$\gamma$J regions was performed as previously described (Gu et al., 1991) on aliquots of the same DNA samples that were used for PCR amplification of $\gamma$HV-68 gp150. Primers specific for the 5′ region of the immunoglobulin D$\gamma$ gene and the 3′ region of the immunoglobulin J$\gamma$1 gene (Gu et al., 1991), ACAAGC-TTCAACGCAAATGCGTGCT and GGGTCTAGACTCTCAGCC-GGCTCCTCAGGG, respectively, were used. DNA (10 ng) was amplified for 40 cycles using an annealing temperature of 56 °C; 10% of the total PCR reaction was then electrophoresed on ethidium bromide-stained agarose gels, and visualized under UV illumination. DNA from epithelial cell preparations showed no amplified products for rearranged D$\gamma$J regions, whereas splenic DNA amplified by PCR was strongly positive for amplified products.

Quantification of lytic virus. The presence of lytic virus was quantified as previously described (Stevenson et al., 1999; Stewart et al., 1998) using a plaque-forming assay. Briefly, isolated splenic leukocytes, or lung or intestinal epithelial cells, were pulse sonicated (Vibra Cell) to release intracellular virus. After sonication, lysates were centrifuged at 19,000 g for 15 min to remove cellular debris. Limiting dilutions of the lysates were then plated on NIH-3T3 monolayers for 1 h followed by washing and overlaying with 0–15% agar (Difco) in RPMI 1640 with 30% FCS. After 5 days, overlays were removed and cell monolayers stained with crystal violet. The number of p.f.u. was quantified in duplicate at several serial dilutions of lysate to assure accuracy.

Infected centres assay. The presence of latent virus was quantified using an infective centres assay as previously described (Cardin et al., 1996; Sunil-Chandra et al., 1992b). For quantification of latent virus, limiting dilutions of isolated splenic leukocytes or lung or intestinal epithelial cells were placed onto monolayers of NIH-3T3 cells. After 24 h, an agar overlay supplemented with media and FCS was added and allowed to incubate for 5 days in 5% CO$_2$. The monolayers were then fixed and stained with crystal violet and the number of infective centres counted in duplicate for several dilutions of cells for each experimental condition.

PCR amplification of $\gamma$HV-68 gp150 DNA. To demonstrate the presence of $\gamma$HV-68 DNA in cells or tissues, a sensitive and specific PCR amplifying the DNA encoding $\gamma$HV-68 gp150 was developed. DNA was extracted from cells or tissues with a QiAmp DNA Mini Kit, according to the manufacturer’s instructions (Qiagen), quantified on DNA Dipsticks, denatured, and precipitated. A nested PCR procedure was developed which consisted of 20 cycles of amplification using the positive and negative strand primers, CCATCTAGCGGTTACACATTCTTACATT and TTTACTGGTCTACCTTTGTTTGGG, respectively; 10% of this PCR amplification was then amplified for 20 cycles using the positive and negative strand primers, CGAACAACATCCACATCAATTATCGC and GTATCTGATGTTGTCAGCAGGAGCGT, respectively, which were derived from the published sequence for $\gamma$HV-68 gp150 (Stewart et al., 1996). The annealing temperature for both PCR amplifications was 63 °C. 10% of the second PCR amplification was then electrophoresed on ethidium bromide-stained agarose gels, and amplified product was visualized under UV illumination. Amplified $\gamma$HV-68 gp150 DNAs were compared to size standards (Promega) electrophoresed on the same gel to assure that a 462 bp fragment was being amplified. Amplified fragments were also isolated (Prep-a-gene, Bio-Rad) and subjected to direct DNA sequencing (fmol Sequencing, Promega) as previously described (Bost et al., 1995; Bost & Mason, 1995) to assure the identity of the $\gamma$HV-68 gp150 coding sequence previously reported (Stewart et al., 1996).

Images of ethidium bromide-stained gels were obtained with a Direct Scan Instant Image Polaroid camera. The images were then scanned into Adobe Photoshop 4.0 using an UMAX Astra 1200 scanner.

Cloning of $\gamma$HV-68 gp150. The DNA encoding $\gamma$HV-68 gp150 was amplified by PCR from viral stocks using the positive and negative strand primers derived from the published sequence (Stewart et al., 1996), TTTCCTGGGGATCACAACATGGTATGCG and AGGTCTCGTCTGGTGAAGGTCACG, respectively. Amplified material was isolated from agarose gels (Prep-a-gene, Bio-Rad), and cloned into the pNoTA/T7 vector using instructions supplied by the manufacturer (Stratagene). Plasmid expressing the cloned fragment was isolated (Del Sal et al., 1988) from E. coli for use as a positive control in PCR and for use in determining the sensitivity of the nested PCR amplification described above.

RT–PCR to detect RNA encoding $\gamma$HV-68 gb. RNA was extracted from isolated epithelial cells using TRIZOL as previously described (Bost & Clements, 1995), treated with DNase, and the RNA re-isolated. To detect the presence of $\gamma$HV-68 RNA encoding the viral gb protein, RNA was reverse transcribed as previously described (Bost & Clements, 1995), and the cDNA was subjected to nested PCR. The gb RNA transcript was selected for analysis since this viral RNA has been shown to be expressed only during the replicative phase of virus infection and not during virus latency (Virgin et al., 1999). The nested PCR for $\gamma$HV-68 gb has been previously described (Virgin et al., 1999) and included amplification for 25 cycles with the positive and negative strand primers, CTGTTCCAAACCACTTTAAC and TTGTTTCTCAGGGCACGTTC, respectively, followed by reamplification of 10% of the first PCR reaction with the positive and negative strand primers, ATGTTACATCAGGCCAACGG and TCTGTTGTCCTTGTTCACAGG. Amplified products were electrophoresed on ethidium bromide-stained agarose gels to show the presence of a 261 bp fragment of $\gamma$HV-68 gb.

Results

Oral and gastric inoculation with $\gamma$HV-68 is an efficient route for viral infection and establishment of latency in the spleen

To determine whether $\gamma$HV-68 introduced into the oral cavity or gastrointestinal tract is infectious, various p.f.u. of virus was given to groups of mice, and the development of the
Fig. 1. Leukocytosis in mice following oral, gastric or intranasal inoculation with γHV-68. Mice were orally, gastrically or intranasally inoculated with the indicated p.f.u. of γHV-68 or with medium only. One group of mice received a ‘mock’ gastric inoculation by intubating and removing a feeding tube containing γHV-68 without injecting the virus. 15 days post-infection, mice were euthanized and splenic leukocyte counts (a) or the number of splenic infective centres (b) determined. In (a), each bar represents mean values ± standard deviations for six mice. In (b), each bar represents infective centres for a single animal. Samples which had fewer than 1 p.f.u. of virus were considered below the level of detectability for this assay. This experiment was performed three times with similar results.

Fig. 2. PCR to detect the DNA encoding γHV-68 gp150 in spleens of mice gastrically or orally inoculated with virus. Mice were orally or gastrically inoculated with the indicated p.f.u. of γHV-68. One group of mice received a ‘mock’ gastric inoculation by intubating and removing a feeding tube containing γHV-68 without injecting the virus. 15 days post-infection, two separate mice per inoculum were euthanized, and splenic DNA was isolated for PCR analyses. Results are shown as amplified fragments electrophoresed on ethidium bromide-stained agarose gels. These experiments were performed three separate times with similar results.

mononucleosis-like disease was followed. Splenomegaly is one hallmark of this disease, and typically peaks 15–17 days post-infection (Cardin et al., 1996). This splenomegaly results from a virus-induced leukocytosis which can be quantified by leukocyte counts from infected mice. Fig. 1(a) clearly shows a marked leukocytosis for orally and gastrically inoculated mice at 15 days post-infection, which was similar to that observed for intranasally infected mice. Further, there was a dose-response relationship between the development of splenomegaly and the amount of virus used for inoculation.

Latent infection of B lymphocytes (Cardin et al., 1996; Sunil-Chandra et al., 1992a), and possibly macrophages (Weck et al., 1999), in peripheral lymphoid organs is also a hallmark of γHV-68 infection. Using an infective centres assay, it was shown that oral and gastric inoculations established latency in splenic lymphocytes at 15 days post-infection (Fig. 1b). Further, as little as 600 p.f.u. of virus was capable of inducing latency in most animals when given orally or intragastrically.

It should also be noted that the results in Fig. 1(b) were due to the presence of latent virus and not the presence of replicating virus. Plaque assays were performed at the same time as infective centre assays using lysates of isolated splenic leukocytes to quantify the presence of replicating virus. Such assays were repeatedly negative (i.e. less than 1 p.f.u.), as is consistent with previous reports for studies using intranasal inoculations of virus (Cardin et al., 1996; Sunil-Chandra et al., 1992a).

The presence of γHV-68 gp150 DNA in splenic lymphocytes following oral and intragastric inoculation of virus was detected using a sensitive, nested PCR. This PCR was approximately 1000 times more sensitive than the infectious centres assay, and could detect the presence of viral DNA using dilutions of γHV-68 preparations which represented 10⁻¹⁰ p.f.u. Furthermore, addition of limiting amounts of cloned γHV-68 gp150 DNA to aliquots of 10 ng of uninfected splenic DNA demonstrated that this nested PCR procedure could detect as few as 10 copies of gp150 DNA. Taken together, these results demonstrated the specificity and sensitivity of this nested PCR procedure.
Gammaherpesvirus-68 infection of gut epithelium

Fig. 3. Development of splenic leukocytosis and virus latency in the spleens of mice following gastric or oral inoculation with \( \gamma \)HV-68. Groups of mice were gastrically or orally inoculated with 60,000 p.f.u. of \( \gamma \)HV-68. At the indicated times post-infection, mice were euthanized and splenic leukocyte counts determined (a). Results are presented as means ± standard deviations of triplicate determinations. Splenic leukocytes were also placed on NIH-3T3 cell monolayers to quantify infective centres per spleen indicating the presence of latent virus (b). Each bar represents the mean infective centres per spleen of individual mice. This entire experiment was performed twice with similar results.

Using this nested PCR, mice gastrically or orally inoculated with various dilutions of \( \gamma \)HV-68 clearly expressed viral DNA in splenic lymphocytes (Fig. 2). Viral DNA was always detected in the spleens of more than 50 mice which were intragastroically or orally inoculated with doses of \( \gamma \)HV-68 as low as 60 p.f.u. Conversely, uninfected mice, or mice receiving a mock gastric inoculation, had no detectable viral DNA present in their splenic lymphocytes (Fig. 2).

**Kinetics of viral infection following gastric and oral inoculation with \( \gamma \)HV-68**

Previous publications (Cardin et al., 1996; Sunil-Chandra et al., 1992a) have established the development of a mononucleosis-like disease and latency following intranasal instillation of \( \gamma \)HV-68 in anaesthetized mice. For comparison, mice were gastrically or orally inoculated with 60,000 p.f.u. of \( \gamma \)HV-68, and groups of animals were euthanized at various times post-infection for analyses. Fig. 3(a) shows that peak leukocytosis was observed at 15 days post-infection, which is similar to previous reports for intranasal infection (Cardin et al., 1996). This was also the peak time for the presence of latent virus in splenic lymphocytes (Fig. 3 b). As noted previously, the results in Fig. 3(b) were not due to the presence of lytic virus. Plaque assays to detect replicating virus in splenic lymphocytes performed at the same time as assays to quantify the presence of lytic virus were repeatedly negative (i.e. less than 1 p.f.u.), consistent with previous reports (Cardin et al., 1996; Sunil-Chandra et al., 1992a). Amplification of \( \gamma \)HV-68 gp150 DNA from splenic leukocytes showed the presence of viral DNA as early as 11 days post-infection, and viral DNA was still present in all infected mice at 23 days following inoculation (data not shown). It should be noted that the development of leukocytosis in gastric and orally inoculated mice by day 15 (Fig. 3) was similar to that reported for intranasally infected mice (Sunil-Chandra et al., 1992a; Tripp et al., 1997).

**Lytic and persistent infection of intestinal epithelial cells following intragastric instillation of \( \gamma \)HV-68**

Recently, it has been suggested that infected lung epithelial cells might serve as a reservoir for persistent \( \gamma \)HV-68 infection following intranasal inoculation (Stewart et al., 1998). We questioned whether intestinal epithelial cells might also serve as a site of virus persistence following intragastric infection. To address this possibility, mice were intranasally or intragastrically inoculated, and epithelial cells were isolated from the lung or intestine at various times post-infection. As shown in Fig. 4(a), mice gastrically intubated with 60,000 p.f.u. of \( \gamma \)HV-68 had replicating virus in intestinal epithelial cell homogenates, but not in lung epithelial cell homogenates, 2 days post-infection. The converse was observed following intranasal inoculation, with only lung epithelial cell homogenates containing replicating virus at 2 days post-infection. These results were supported by the presence of \( \gamma \)HV-68 DNA in epithelial cells only at the site of inoculation (Fig. 4b).
Fig. 4. Quantification of lytic virus in lung or intestinal epithelial cells following intragastric or intranasal inoculation with \( \gamma \)HV-68. Mice were gastrically or intranasally inoculated with 60,000 p.f.u. of \( \gamma \)HV-68, and epithelial cells were isolated from the lung and intestine 2 days post-infection. For (a), limiting dilutions of epithelial cell lysates were placed on NIH-3T3 cells to quantify the presence of replicating virus. Results are presented as means \( \pm \) standard deviations of triplicate determinations. Samples which had fewer than 1 p.f.u. of virus were considered below the level of detectability for this assay. For (b), DNA isolated from each epithelial cell population was subjected to nested PCR analysis to detect the presence of \( \gamma \)HV-68 gp150 DNA, or the cellular gene G3PDH. Following PCR, 10% of the reaction was electrophoresed on ethidium bromide-stained agarose gels, and visualized under UV illumination. Results from two separate mice are shown. These experiments were performed twice with similar results.

To address whether intestinal epithelial cells were persistently infected following oral inoculation of \( \gamma \)HV-68, a kinetics analysis of the presence of lytic virus, viral DNA and viral RNA was performed. Fig. 5(a) shows replicating virus being detected early after inoculation in intestinal epithelial cells early after infection. The amount of lytic virus declined over time and was not detectable at 30 days-post-infection. Despite the inability to detect lytic virus at day 30 post-infection, it was clear that viral DNA encoding gp150 (Fig. 5(b)) and viral RNA encoding gB (Fig. 5(c)) were present in intestinal epithelial cells. Infective centre assays performed at the same time as the lytic assays showed no detectable latent virus in these intestinal epithelial cells at days 15 and 30 post-infection (data not shown). Taken together, these results strongly suggest that intestinal epithelial cells were persistently infected with \( \gamma \)HV-68 following gastric inoculation.

Fig. 5. \( \gamma \)HV-68 persists in intestinal epithelial cells following intragastric inoculation. Mice were gastrically or intranasally inoculated with 60,000 p.f.u. of \( \gamma \)HV-68, and epithelial cells were isolated from the intestine at the indicated times post-infection. For (a), limiting dilutions of epithelial cell lysates were placed on NIH-3T3 cells to quantify the presence of replicating virus. Results are presented as means \( \pm \) standard deviations of triplicate determinations. Samples which had fewer than 1 p.f.u. of virus were considered below the level of detectability for this assay. For (b), DNA isolated from each population of epithelial cells was subjected to nested PCR analysis to detect the presence of \( \gamma \)HV-68 gp150 DNA, or the cellular gene G3PDH. Following PCR, 10% of the reaction was electrophoresed on ethidium bromide-stained agarose gels, and visualized under UV illumination. Results from two separate mice are shown for each time-point. For (c), total RNA was extracted from isolated epithelial cells and subjected to nested RT–PCR analyses to detect viral transcripts encoding \( \gamma \)HV-68 gB. Following RT–PCR, 10% of the reaction was electrophoresed on ethidium bromide-stained agarose gels, and visualized under UV illumination. Results from three separate mice are shown for 15 days post-infection (lanes 1, 2 and 3) and for 30 days post-infection (lanes 5, 6 and 7). Lanes 4 and 8 represent control reactions, where aliquots of RNA were processed in an identical manner with the exception that reverse transcriptase was omitted from the cDNA reactions. These experiments were performed twice with similar results.

Discussion

The results presented here clearly demonstrate that \( \gamma \)HV-68 can survive the harsh environment of the gastrointestinal tract to efficiently infect intestinal epithelial cells. Following this
productive infection, the virus disseminates from this mucosal surface, presumably via the draining lymph nodes, to cause a peripheral mononucleosis-like disease. It is clear from the results presented here that bypassing the oral cavity does not substantially hinder the ability of γHV-68 to cause systemic disease. This was a surprising result since this gammaherpesvirus is routinely inoculated intranasally. Previous work (Blaskovic et al., 1984; Rajcani et al., 1985) demonstrated that newborn or suckling mice could be infected orally with γHV-68; however, the present study is the first to demonstrate that fully immunocompetent adult mice can be efficiently infected following oral inoculation. Further, this is the first study to demonstrate that bypassing the oral cavity results in a productive infection of intestinal epithelial cells with subsequent progression to systemic disease.

It would be unlikely that γHV-68 could so readily infect mice via the gut epithelium if this virus was not adapted to do so. Therefore, these results suggest the possibility that a normal and common route of transmission for this virus might be via a productive infection of the intestinal epithelium. One question which remains unanswered from the present studies is the predominant site for infection of epithelial cells following oral inoculation with γHV-68. Since epithelial cells within the oral cavity as well as the gut were exposed to the virus following oral inoculations, it will be important in future studies to identify sites of productive virus replication.

The ability of γHV-68 to efficiently infect intestinal epithelial cells provides an intriguing model for the study of viral pathogenesis and the host response at a mucosal surface. Several in vitro studies using human intestinal epithelial cells have suggested that these cells can be directly infected by a human gammaherpesvirus (Tajima et al., 1998; Takasaka et al., 1998; Yanai et al., 1997), and direct infection of murine intestinal epithelial cells following gastric intubation with γHV-68 has been demonstrated here. However, it is also possible that the spread of virus during the replicative phase occurs predominately by virus entry across basolateral surfaces of gut epithelial cells, as has been suggested by Imai et al. (1998). The spread of virus via basolateral surfaces of epithelial cells would likely involve interaction with intraepithelial lymphocytes, which are predominantly CD8+ T lymphocytes. Since this T lymphocyte subset is dysregulated during the systemic mononucleosis phase (Cardin et al., 1996; Tripp et al., 1997), it will be important to determine what effect this viral infection has on the regulated immune response to normal gut flora, and on the maintenance of oral tolerance to most ingested antigens.

It is also clear from the studies presented here that gastric intubation is an efficient route of inoculation for establishment of a systemic leukocytosis. Dissemination of virus to lymph nodes draining the small intestines could occur following infection of epithelial cells, but could also occur via virus entry into the Peyer’s patches as M cells sample gut immunogens. Intranosal infection does not result in significant numbers of latently infected B lymphocytes within the mesenteric lymph nodes (Sunil-Chandra et al., 1992a), suggesting that the route of inoculation may influence the compartment of B lymphocytes, and possibly macrophages, which harbour the virus. It is not clear at present whether the number of latently infected cells present in the Peyer’s patches or mesenteric lymph nodes will be significantly increased if the route of exposure to the virus is via the gut.

The recent observation that epithelial cells may serve as a reservoir for persistent virus (Stewart et al., 1998) is supported by the studies performed here. At 30 days post-infection following intragastric inoculation, intestinal epithelial cells contained viral DNA and RNA (Fig. 5b, c) in the absence of any detectable replicating virus (Fig. 5a). The possibility of persistently infected intestinal epithelial cells may have implications for human disease. For example, gammaherpesvirus-related gastrointestinal diseases (Shinohara et al., 1998; Tokunaga et al., 1993; Yanai et al., 1997) may occur preferentially in patients with persistent viral infection of intestinal epithelial cells.

The presence of populations of gut epithelial cells which are persistently infected with γHV-68 may also have important clinical implications for the development of virus-associated cancers (Tokunaga et al., 1993). Intranasal and intravenous administration of γHV-68 to mice resulted in a significant portion of these animals developing lymphoproliferative disease (Sunil-Chandra et al., 1994). Gammaherpesvirus-associated lymphoproliferative diseases in humans are also well documented (Young et al., 1989), and this correlation supports the use of γHV-68 as a model for investigating the mechanisms associated with gammaherpesvirus-induced cellular transformation. In addition, EBV is known to associate with cancers of epithelial cell origin, including nasopharyngeal (Glaser et al., 1976; Klein et al., 1974; Nonoyama et al., 1973; Wolf et al., 1973) and gastric (Gulley et al., 1996; Selves et al., 1996; Tokunaga et al., 1993) carcinomas. In the light of our studies (Fig. 5) and recent investigations demonstrating persistent, or possibly latent, infection of lung epithelial cells (Stewart et al., 1998), it is tempting to speculate that cells (i.e. B lymphocytes or epithelial cells) which can be latently infected with a gammaherpesvirus have an increased susceptibility for transformation. Correlations of the role of EBV in gastric carcinomas have been supported by studies demonstrating that EBV can infect intestinal epithelial cells (Gulley et al., 1996; Imai et al., 1994; Tajima et al., 1998; Takasaka et al., 1998; Yanai et al., 1997), and that EBV is associated with some inflammatory bowel diseases (Grotsky et al., 1971; Ruther et al., 1998; Wakefield et al., 1992). Studies to assess whether the development of gastric carcinomas or inflammatory bowel diseases can be augmented following introduction of γHV-68 directly into the gut can now be performed.

Taken together, the studies presented here demonstrate a surprising ability of γHV-68 to survive the acidic and...
proteolytic environment of the upper gastrointestinal tract, and to productively infect intestinal epithelial cells. The rapid development of systemic mononucleosis-like disease following intragastric administration suggests a normal route of infection for this pathogen in these rodents. Based on our studies, γHV-68 infection of the gut mucosa should be an intriguing model system for investigating viral pathogenesis at a mucosal surface and for exploring the possibility of cellular transformation of intestinal epithelial cells.

References


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