Murine gammaherpesvirus-68 glycoprotein H–glycoprotein L complex is a major target for neutralizing monoclonal antibodies

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Herpesviruses characteristically persist in immune hosts as latent genomes, but to transmit infection they must reactivate and replicate lytically. The interaction between newly formed virions and pre-existing antibody is therefore likely to be a crucial determinant of viral fitness. Murine gammaherpesvirus-68 (MHV-68) behaves as a natural pathogen of conventional, inbred mice and consequently allows such interactions to be analysed experimentally in a relatively realistic setting. Here, monoclonal antibodies (mAbs) were derived from MHV-68-infected mice and all those recognizing infected-cell surfaces were tested for their capacity to neutralize MHV-68 virions. All of the neutralizing mAbs identified were specific for the viral glycoprotein H (gH)–gL heterodimer and required both gH and gL to reproduce their cognate epitopes. Based on antibody interference, there appeared to be two major neutralization epitopes on gH–gL. Analysis of a representative mAb indicated that it blocked infection at a post-binding step – either virion endocytosis or membrane fusion.

INTRODUCTION

Herpesviruses are ubiquitous, persistent pathogens. They are usually latent or cell-associated within their hosts and so are primarily controlled by T cells (Yewdell & Hill, 2002; Stevenson, 2004). Antibodies can limit virus replication by Fc receptor-dependent cytotoxicity (Balachandran et al., 1982), but generally fail to prevent disease when T-cell function is impaired. However, antibodies also confront herpesviruses when they exit immune hosts. Here, viral genomes are packaged into discrete infectious particles rather than being cell-associated and may therefore be susceptible to neutralization.

It has long been known that immune sera can neutralize herpesviruses in vitro (Fenner et al., 1974). A major advance in understanding this neutralization came with the use of monoclonal antibodies (mAbs) to delineate individual neutralization targets. The majority of neutralizing mAbs described to date recognize viral attachment proteins and prevent virions from binding to their cellular receptors. Classic targets include the herpes simplex virus (HSV) glycoprotein D (gD) (Para et al., 1985) and the Epstein–Barr virus (EBV) gp350 (Thorley-Lawson & Poordry, 1982). mAbs have also been described that block viral membrane fusion, usually by binding to the conserved herpesvirus gH (Miller & Hutt-Fletcher, 1988; Fuller et al., 1989; Gompels & Minson, 1989; Liu et al., 1993; Urban et al., 1996). Such mAbs appear to be rarer than those that block binding, but there has been little quantitative analysis of neutralizing-antibody responses. This is an important goal, as major neutralization targets should be optimal antigens for vaccine-mediated infection control. For all the potential of antibodies to neutralize herpesviruses, the typical outcome of infection is long-term viral transmission.

Our understanding of gammaherpesvirus-specific antibody responses has been constrained by the limited host ranges of these viruses and by the frequent difficulty of propagating them lytically in vitro. Murine gammaherpesvirus-68 (MHV-68), unlike EBV and Kaposi’s sarcoma-associated herpesvirus (KSHV), readily undergoes lytic replication in vitro and therefore provides a convenient model for analysing antibody responses. The natural host of MHV-68 is probably the yellow-necked mouse (Kozuch et al., 1993). However, it also behaves as a natural pathogen in inbred laboratory mouse strains, persisting without causing disease unless the host is immunodeficient (Speck & Virgin, 1999; Stevenson & Efstathiou, 2005). As MHV-68 can harness the tools of murine immunobiology for pathogenesis studies, it has become a major focus of gammaherpesvirus research. The molecular mechanisms of immune control and virus evasion are consequently now better understood for MHV-68 than for any other gammaherpesvirus.

We aimed to address, using MHV-68, the question of why antibody does not normally neutralize the virions of gammaherpesviruses when they are shed from persistently
infected hosts and whether this could be remedied. Although the MHV-68 gp150 (encoded by the M7 gene) and ORF4 proteins have both been reported to elicit neutralizing polyclonal rabbit sera (Stewart et al., 1999; Gangappa et al., 2002), these findings are somewhat at odds with the fact that neither protein is essential for lytic virus replication (Adler et al., 2002), these findings are somewhat at odds with the fact that neither protein is essential for lytic virus replication (Adler et al., 2002; de Lima et al., 2004). It is possible that gp150-or ORF4-specific immune serum inhibits infection in vitro by steric hindrance or by cross-linking virions. Because immune sera incorporate complex mixtures of antibody specificities, affinities and isotypes, such phenomena are hard to dissect. Reproducing the neutralizing activity of immune sera with mAbs would therefore seem an obvious way to proceed. However, as yet, no MHV-68-neutralizing mAbs have been described.

The most likely MHV-68 targets for neutralization are gB and gH. Both are essential proteins (Moorman et al., 2004; Song et al., 2005) that are conserved in all mammalian herpesviruses. gB, gH and gL have all been described as neutralization targets for KSHV (Akula et al., 2002; Naranatt et al., 2002). gM is also conserved and essential (May et al., 2005a), but is less likely to be a neutralization target, as it is confined largely to the plane of the virion membrane and functions mainly in virion egress (Lake & Hutt-Fletcher, 2000). MHV-68 gB is a virion protein (Lopes et al., 2004) and is probably analogous to the receptor-binding gB of KSHV (Akula et al., 2002). However, a small survey of MHV-68 gB-specific mAbs did not identify any with neutralizing activity (Lopes et al., 2004).

Herpesvirus gH typically forms a heterodimer with another conserved glycoprotein, gL (Hutchinson et al., 1992; Yaswen et al., 1993; Lomonte et al., 1997; Naranatt et al., 2002). Several herpesviruses also encode an optional third component of the gH–gL complex that modulates its function (Huber & Compton, 1998; Wang et al., 1998; Mori et al., 2004). The paradigm is EBV, which uses gH–gL to infect epithelial cells and gH–gL–gp42 to infect B cells (Wang et al., 1998). Relatively little is known about gH and gL of gamma-2 herpesviruses beyond the fact that they are virion components (Lomonte et al., 1997; Naranatt et al., 2002). The gH–gL-specific antibodies that are elicited by natural infection and the gH–gL epitopes that are targets for neutralization are unknown. Here, we have identified the MHV-68 gH–gL heterodimer as a major target for neutralizing mAbs derived from MHV-68-infected mice. All of the gH–gL-specific neutralizing mAbs identified required both proteins for the expression of their cognate epitopes. Analysis of a representative gH–gL-specific mAb indicated that it blocked viral entry at a post-binding step.

**Methods**

**Mice.** Female BALB/c mice were purchased from Harlan UK Ltd, housed in the Cambridge University Department of Pathology and infected intranasally at 6–12 weeks old with 2 × 10⁶ p.f.u. MHV-68. At 3–5 months post-infection, they were given an intraperitoneal boost of 5 × 10⁹ p.f.u. MHV-68. Spleens were harvested 3 days later.

**Viruses.** To create fluorescently tagged MHV-68, 1·5 kb genomic flanks around genomic co-ordinate 70915 were amplified by PCR (Hi-Fidelity PCR kit; Roche Diagnostics) and cloned into pSP73 (Promega). The enhanced yellow fluorescent protein (eYFP) coding sequence was then cloned between the flanks to make an extended cytoplasmic tail on gp150. eYFP plus its genomic flanks was subcloned into the pST76K-SR shuttle vector and recombined into an MHV-68 genomic bacterial artificial chromosome (BAC) by standard protocols (Adler et al., 2000). Infectious virus was reconstituted by transfecting BAC DNA into BHK-21 cells with Fugen-6 (Roche Diagnostics). The loxP-flanked BAC cassette was removed by passaging virus through NIH-3T3-CRE cells (Stevenson et al., 2002). All viruses were grown and titrated in BHK-21 cells (Coleman et al., 2003). Virus stocks were prepared from infected BHK-21 cell supernatants by ultracentrifugation. Virions were purified from virus stocks by ultracentrifugation on Ficoll gradients (May et al., 2005b).

**Cells and antibodies.** BHK-21, NIH-3T3-CRE, NS0, 293T, MRC-5 and CHO-K1 cells were propagated as described previously (May et al., 2005b). B-cell hybridomas were generated by fusing splenocytes with NS0 cells (Galfré & Milstein, 1981) using polyelectrolyte glycol 1500 (Roche Diagnostics). Hybrids were selected with asearsine (1 μg ml⁻¹/hypoxanthine (100 μM) and cultured in 20% fetal calf serum on irradiated MRC-5 feeder cells. Subcloned hybrids were then weaned onto 5–10% fetal calf serum without feeders. mAbs were concentrated from hybridoma supernatants by ammonium sulfate precipitation and quantified by ELISA against mouse IgG standards. The mAbs referred to in this study are listed in Table 1. mAbs were tested for neutralization by incubating either purified antibody or 1–3 ml hybridoma supernatant with 100 p.f.u. MHV-68.

**Plasmids.** MHV-68 gH (ORF22, genomic co-ordinates 34833–37022) and gL (genomic co-ordinates 65437–65027) coding sequences were cloned upstream of the enhanced green fluorescent protein (eGFP) coding sequence in pEGFP-N2 (Clontech) to add C-terminal eGFP. To make glycosylphosphatidylinositol (GPI)-linked derivatives, the gH extracellular domain (genomic co-ordinates 34833–36941) or gL (genomic co-ordinates 65437–65027) was cloned upstream of a human decay-accelerating factor (gPI)-attachment site, as described previously (Lopes et al., 2004). The gl coding sequence (genomic co-ordinates 65437–65024) and the larger genomic ORF47 (genomic co-ordinates 65545–65024) were also cloned into pcdNA3 (Invitrogen). Each genomic segment was amplified by PCR (Hi-Fidelity PCR kit; Roche Diagnostics), including restriction-enzyme sites in the primers compatible with sites in the vector. The 5’ end of the gl mRNA was mapped by RACE (5’/3’ RACE kit; Roche Diagnostics). The RACE primers matched genomic co-ordinates 65151–65170 for cDNA synthesis, 65191–65210 for PCR amplification [paired with a primer matching the 5’ poly(A) cDNA tail added by terminal deoxynucleotide transferase] and 65230–65250 for a second round of PCR amplification. The PCR products were sequenced to locate the 5’ poly(A) tail.

**Biochemical analysis.** Metabolic labelling with [³⁵S]cysteine/methionine, glycoprotein immunoprecipitation and immunoblotting were performed as described previously (Boname & Stevenson, 2001; May et al., 2005b). eGFP was identified with a rabbit anti-GFP polyclonal antibody (Santa Cruz Biotechnology). For biochemical analysis, gH was immunoprecipitated from purified virions with mAb T4C5, resolved by SDS-PAGE, fixed in 50% methanol/10% acetic acid and stained with Coomassie R250 (Sigma) or silver nitrate. Coomassie-stained bands were excised for matrix-assisted
**RESULTS**

**Recovery of MHV-68-neutralizing mAbs from MHV-68-infected mice**

MHV-68 allows mAbs to be recovered readily from a natural host. We generated B-cell hybridomas from BALB/c splenocytes 3–5 months post-infection. As MHV-68 virions are found on the plasma membrane of infected BHK-21 cells (de Lima et al., 2004), all virion-binding mAbs also should bind to infected cells. Therefore, we tested all hybridoma supernatants that recognized unfixed, MHV-68-infected BHK-21 cells for neutralization. Typical neutralization data are shown in Fig. 1(a). mAbs that neutralized MHV-68 for BHK-21 cell infection also neutralized it for infection of murine embryonic fibroblasts and NS0 myeloma cells (data not shown).

### Neutralizing mAbs immunoprecipitate MHV-68 gH

We screened > 300 MHV-68-infected cell surface-specific hybridoma supernatants for neutralization. Less than 5 % of these gave > 50 % plaque reduction. We tested the antigen specificity of neutralizing hybridomas by immunoprecipitating 35S-labelled viral proteins from MHV-68-infected BHK-21 cell lysates. All precipitated a 90 kDa band that was distinct from gB (Fig. 1b; Lopes et al., 2004), ORF27 (May et al., 2005c), ORF58 (May et al., 2005d), ORF28 (May et al., 2005b), gM, gN (May et al., 2005a) and ORF4 (Fig. 1b and c). To identify this band, we immunoprecipitated it from unlabelled virion lysates by using the neutralizing mAb T4C5 (Fig. 1d) and analysed it by mass spectrometry and N-terminal sequencing (Fig. 1e). This established that the 90 kDa band was MHV-68 gH.

**gH and gL are necessary and sufficient to generate the epitopes recognized by neutralizing mAbs**

Antibody specificity for gH was tested further by staining cells transfected with a gH-expression plasmid (Fig. 2). In order to reduce any toxicity of gH, we replaced its transmembrane domain and cytoplasmic tail with the GPI anchor of human decay-accelerating factor. gH–GPI was expressed at the cell surface (mAb 8C1 in Fig. 2a), but was detected by only 3/35 of the mAbs that immunoprecipitated gH from infected cells (see mAb 7D6 in Fig. 2a). Therefore, gH–GPI alone adopted a conformation different from that of gH on infected cells.

As HSV (Gompels & Minson, 1989; Hutchinson et al., 1992) and EBV gH (Yaswen et al., 1993) misfold in the absence of gL, we hypothesized that the altered antigenicity of gH–GPI reflected a lack of gL. In order to reconstitute the putative MHV-68 gH–gL heterodimer, first we had to identify the N terminus of gL. One annotation of the MHV-68 genome (GenBank accession no. U97553) places the 5′ methionine of gL at the start of ORF47 (genomic co-ordinate 65545); another (GenBank accession no. AF105037) places it at the fifth in-frame ORF47 methionine (genomic co-ordinate 65437) to give a more conventional N-terminal signal sequence. By using RACE, we identified the 5′ end of the ORF47 transcript in MHV-68-infected BHK-21 cells as making genomic co-ordinate 65437 the 5′ AUG, in agreement with genomic co-ordinate 65488/65489 (Fig. 2b). This would make genomic co-ordinate 65437 the 5′ AUG, in agreement

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**Table 1. mAbs used in this study**

<table>
<thead>
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<th>mAb</th>
<th>Specificity*</th>
<th>Isotype</th>
<th>Neutralization</th>
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</tr>
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<tr>
<td>T7H9</td>
<td>gB</td>
<td>IgG2a</td>
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* All gH-specific and gH–gL-specific mAbs precipitated a 90 kDa band from infected-cell lysates and recognized 293T cells transfected with gH + gL. ORF4-specific mAbs recognized CHO-K1 cells transfected with an ORF4 expression plasmid and BHK-21 cells infected with wild-type MHV-68, but not BHK-21 cells infected with an ORF4 knockout virus (Adler et al., 2000). gH-specific mAbs additionally recognized 293T cells transfected with gH only. mAbs against gp150 (de Lima et al., 2004), gB (Lopes et al., 2004) and gN (May et al., 2005a) have been described previously.
Co-expressing gH–GPI with the RACE-mapped gL reconstituted the epitopes recognized by all gH–gL-specific mAbs (7D6 in Fig. 2a). In contrast, co-expressing gH–GPI with the entire genomic ORF47 failed to reconstitute any more epitopes than gH–GPI alone (data not shown). We concluded that genomic co-ordinate 65437 corresponds to the N-terminal methionine of the gL precursor.

No gH–gL-specific mAbs recognized hL attached to the cell surface by a GPI anchor, even though this protein was recognized by an MHV-68-immune rabbit serum (Fig. 2c). Also, no gH–gL-specific mAbs recognized a glutathione S-transferase–gL fusion protein by ELISA (data not shown). Interestingly, gL–GPI was at least as effective as soluble gL in restoring gH–gL epitopes to gH–GPI (Fig. 2d), implying that gL lies close to the virion membrane in the gH–gL heterodimer. Thus, it may be relatively inaccessible to antibody.

mAbs specific for gH–gL precipitate two virion glycoproteins

To identify MHV-68 proteins associated with mature gH, we infected BHK-21 cells, labelled the cells with [35S]cysteine/methionine, chased the label overnight and recovered labelled virions from supernatants. Virion gH immunoprecipitates were then analysed by SDS-PAGE, with or without reduction and with or without N-glycanase treatment (Fig. 3). mAbs 7D6 and T2C12 precipitated gH as a 90 kDa band that was N-glycosidase F (PNGase F)-sensitive and endoglycosidase H (endoH)-resistant (Fig. 3a). Virion gH therefore had complex or hybrid N-linked glycans. In control lanes, virion ORF4 had only a small N-linked glycan. The disulphide-linked 65 and 55 kDa fragments of virion gB (Lopes et al., 2004) both retained significant endoH sensitivity.
Three smaller bands co-precipitated specifically with gH. These were resolved on higher-percentage gels (Fig. 3b). The two larger bands (~19 kDa) were PNGase F-sensitive and therefore distinct from the similar-sized bands co-precipitating with gB. The smallest band (13 kDa) did not appear to be N-glycosylated. None was disulphide-linked to gH. These proteins did not yield interpretable mass-spectrometry fragments. However, it seemed likely that at least one of them was gL, which has a predicted unglycosylated molecular mass of 13 kDa. PNGase F treatment reduced both of the larger bands to exactly the size of the smallest band (Fig. 3b). A minimal interpretation would therefore be that the two larger bands were different N-linked glycoforms of gL and the smallest band was gL lacking N-linked glycans. A 20 min label of infected cells followed by immunoprecipitation (Fig. 3c) established that they associated with gH in the endoplasmic reticulum, soon after or coincident with protein synthesis.

gH associates with eGFP-tagged gL in transfected cells

To identify gL directly, we tagged it with C-terminal eGFP (gL–eGFP). We also tagged full-length gH in the same way (gH–eGFP). Combinations of gH and gL were then transfected into 293T cells (Fig. 4). Flow cytometry established that gH–eGFP reached the cell surface in the absence of gL (mAb 8C1), but did not reproduce the epitopes recognized by neutralizing mAbs such as T7G7 (Fig. 4a). gH–GPI behaved similarly (Fig. 4b). The higher mAb 8C1 staining of eGFP+ cells after transfection with gH–GPI + eGFP in Fig. 4(b) presumably reflects more efficient transfection with both plasmids. Thus, it was not possible to interpret different levels of mAb staining, only whether there was staining or not. The key finding was that mAb T7G7, like mAb 7D6 in Fig. 2(a), recognized gH–eGFP only when gL or gL–eGFP was also expressed.

Immunofluorescence (Fig. 4c) established that, even in intracellular sites, gH expressed without gL was recognized by gH-specific mAbs such as 8C1, but not by the majority of gH-precipitating mAbs, represented here by 7D6 and T4C5. Their epitopes were again restored by gL, consistent with gH and gL associating in the endoplasmic reticulum to form the fully folded heterodimer (Fig. 3c).

Confocal microscopy (Fig. 4d) established that gL–eGFP and gH–GPI co-localized in transfected cells, implying that they were physically associated. gL–eGFP was relatively unstable in transfected cells – the major eGFP band on immunoblots was free eGFP rather than the fusion protein (data not shown). Nevertheless, we could still show that, when gH–eGFP or gH–GPI was present, a gH–gL-specific mAb co-precipitated gL–eGFP (Fig. 4e). gH–eGFP was hard to identify, but appeared to be precipitated by mAb 7D6 only when gL–eGFP was also present.

Neutralizing mAbs recognize only a limited number of gH–gL epitopes

We used biotinylated mAbs to map by mutual interference the domains on gH–gL recognized by different neutralizing mAbs (Fig. 5a and b). The best neutralizing mAbs formed two groups, with mAbs from independent fusions in each group. Thus, there may be as few as two distinct neutralization epitopes on gH–gL. mAbs that recognized gH–GPI
alone (T6D11 in Fig. 5b) did not overlap in specificity with those recognizing gH–gL. Also, they did not neutralize, although the number of mAbs tested was small (n = 3).

There was little evidence for synergism between mAbs recognizing different gH–gL neutralization epitopes (Fig. 5c).

Neutralization by a gH–gL-specific mAb occurs after cell binding

Gammaherpesviruses have been reported variously to fuse with plasma membranes and endosomal membranes (Miller & Hutt-Fletcher, 1992; Akula et al., 2003). MHV-68 infection of BHK-21 cells was inhibited by inhibitors of endocytosis or lysosomal acidification (Fig. 6). Fusion therefore appeared to occur intracellularly. We used MHV-68 with eYFP-tagged gp150 (M7–eYFP) to define the point of entry blocked by gH–gL-specific neutralization (Fig. 7). Both M7–eYFP-infected cells (Fig. 7a) and M7–eYFP virions (Fig. 7b) were fluorescent. We quantified virion binding to cells by flow cytometry (Fig. 7c). In a 4 h infection, cycloheximide treatment largely abolished cellular fluorescence that depended on viral eGFP expression (Fig. 7d). It had no effect on the fluorescence transferred to cells by the M7–eYFP virus, which therefore required only binding. Immune serum or soluble heparin blocked this fluorescence transfer.

Next, we compared the capacity of immune serum or the gH–gL-specific, neutralizing mAb 7D6 to inhibit cell binding and plaque formation by M7–eYFP MHV-68 (Fig. 7e).

In a 2 h infection, at least 3 p.f.u. per cell was required to detect cellular fluorescence by flow cytometry (data not shown). This high level of input virions made complete neutralization difficult. Nonetheless, we could compare the inhibition of binding by serum and mAb doses that gave similar levels of plaque reduction. Both 7D6 and the control, non-neutralizing, gp150-specific mAb T1A1 gave a minor, non-titrating reduction in cell binding. As this was not associated with reduced plaque formation by T1A1, it presumably reflected a non-specific effect of antibody binding. The key finding was that immune serum gave a good correlation between reduced cell binding and reduced plaque formation, whereas mAb 7D6 did not. Indeed, with 7D6, there tended to be an inverse correlation. gH–gL-directed neutralization therefore did not block cell binding.

Neutral (pH 7) washing leaves protein interactions intact and so removes only those virions that are not attached to cells, whereas acid (pH 3) washing denatures proteins and so removes all non-endocytosed virions. Thus, by comparing pH 3 and pH 7 washes with untreated M7–eYFP virus (Fig. 7f). Virion/serum mixtures also showed little difference between washes, consistent with immune serum blocking the attachment of virions to cells. However, virion/
7D6 mixtures remained noticeably more sensitive to acid than to PBS washing. Therefore, it appeared that mAb 7D6 allowed cell binding, but inhibited endocytosis or membrane fusion. It was not possible to distinguish these processes, as non-endocytosed virions would not fuse (Fig. 6) and endocytosed, non-fusing virions may be recycled back to the cell surface. The main conclusion was that gH–gL-specific neutralization inhibited events downstream of cell binding.

**DISCUSSION**

The interactions between herpesviruses and antibodies continue to pose significant questions, most notably in the discrepancy between efficient viral neutralization *in vitro* and the failure of natural antibody responses to prevent viral transmission *in vivo*. Passive antibody transfer can limit *in vivo* herpesvirus replication, but this does not imply...
neutralization, as antibody-dependent cytotoxicity is a more important defence against cell-associated viruses. A key role for neutralizing antibody comes when viral genomes leave their host as cell-free virions. Neutralization-based interventions are therefore appropriate for controlling infection in populations rather than in individuals. One approach might be to establish strong neutralizing-antibody responses in virus carriers by post-exposure vaccination and so stop them transmitting infection. A crucial task is to define, by using mAbs, possible molecular targets for such vaccination, and this is what we have sought to do with MHV-68. All of our MHV-68-neutralizing mAbs recognized the gH–gL heterodimer. In contrast to immune sera, which blocked virion binding to cells, gH–gL-directed neutralization blocked at a post-binding step, either endocytosis or membrane fusion.

Our failure to identify neutralizing mAbs that blocked cell binding may reflect the fact that MHV-68 has alternative binding targets, so that single antibodies are never enough. Incoming virions probably bind first to glycosaminoglycans (GAGs) (Fig. 7c; de Lima et al., 2004) and MHV-68 may – like other herpesviruses – encode multiple GAG-binding proteins. This highlights a general shortcoming of blocking cell binding: herpesviruses encode multiple adhesion proteins, different receptors may be used on different cell types and the primary target cell generally may not be known and may not be unique. Thus, gp350-specific mAbs can neutralize EBV virions for B-cell infection (Thorley-Lawson & Pookey, 1982), but epithelial cells are probably the primary target for EBV in new hosts and gp350 is dispensable for epithelial-cell infection (Janz et al., 2000). Once an epithelial cell has been infected, subsequent cell-to-cell dissemination is probably hard for antibodies to stop.

Every entry pathway converges on membrane fusion, so this may be a more universally effective neutralization target. By analogy with other herpesviruses, membrane fusion is likely to be a function of the MHV-68 gH–gL complex and therefore is likely to be the process inhibited by gH–gL-specific neutralizing mAbs. If fusion involves a pH-dependent conformational change in gH–gL following endocytosis, mAbs may act to stabilize the native virion conformation. They may also prevent gH–gL from interacting with a cellular co-receptor (Molesworth et al., 2000; Parry et al., 2005). The neutralizing mAbs presumably bind to gH. gL is only a small protein; it is approximately one-third N-linked glycan by mass (Fig. 3b) and probably is sited close to the virion membrane in the heterodimer (Fig. 2d). An eGFP tag on gL did not affect its binding to gH, implying that eGFP lay on the outer surface of gH–gL, but neither did it affect gH–gL recognition by mAbs. Therefore, there would seem to be little room left on gL for antibody to bind. Detailed analysis of the neutralization mechanism must await resolution of the gH–gL structure. The fact that the best neutralizing mAbs recognized only two distinct epitopes suggests that gH–gL has few vulnerable points. Even these
Fig. 7. Neutralization and cell binding. (a) BHK-21 cells were infected (2 p.f.u. per cell, 18 h) with wild-type (WT) MHV-68 or MHV-68 with eYFP-tagged gp150 (M7–eYFP). Cells were fixed in methanol, permeabilized in PBS/0.1% Tween 20 and stained for gp150 with mAb T7F5 (red). eYFP fluorescence appeared green and co-localization yellow. (b) BHK-21 cells were infected (3 p.f.u. per cell, 1 h, 37°C) with M7–eYFP virus. Cells were fixed in 4% paraformaldehyde and eYFP expression was visualized by confocal microscopy (green). Actin was counterstained with phalloidin–TRITC (red). (c) BHK-21 cells were either left uninfected or infected (2 h, 37°C) with M7–eYFP virus. Cells were then washed, trypsinized and analysed for eYFP expression by flow cytometry. (d) eGFP-expressing (eGFP+) or eYFP-tagged (M7–eYFP) MHV-68 virions were pre-incubated with medium only (Nil), 5% immune serum (Imm) or 100 μg heparin (Hep) ml⁻¹. BHK-21 cells were then left uninfected (UI) or exposed to the virus preparations as indicated, with or without 100 μg cycloheximide (CHX) ml⁻¹ to block viral protein synthesis. After 4 h, cells were assayed for green-channel fluorescence (eGFP or eYFP) by flow cytometry. (e) M7–eYFP virions were incubated (1 h, 37°C) with dilutions of immune serum (from 2%) or mAb (from 200 μg) and then used to infect BHK-21 cells. UI, Uninfected cells; VIR, cells with untreated virus. Virus titres were measured by plaque assay of a nominal 150 p.f.u. virus. For fluorescence measurements, cells were exposed to virus (m.o.i. = 4) for 2 h at 37°C, washed with PBS and analysed by flow cytometry. mAb 7D6 is neutralizing and specific for gH–gL; mAb T1A1 is non-neutralizing and specific for gp150. (f) M7–eYFP virus was incubated (1 h, 37°C) with serum or mAb 7D6 dilutions as indicated. BHK-21 cells then were exposed to a virus/antibody mixture (2 h, 37°C), washed with PBS or citrate/phosphate buffer (pH 3, acid) and assayed for yellow fluorescence by flow cytometry. UI, Uninfected cells; VIR, cells infected with untreated virus. Each of (c)–(f) is representative of three replicate experiments.
epitopes may be related, as distinct neutralizing mAbs showed very limited synergy.

The requirement for efficient transmission between hosts must be a major force in viral evolution. Thus, it is to be expected that herpesviruses make every effort to evade neutralization. Although neutralization was possible with gH–gL-specific mAbs, it was inefficient: typically, 100 μg antibody was required to neutralize 100 p.f.u. virus. The number of gH–gL complexes per virion is unknown, but this is almost certainly a large molar excess of antibody, perhaps of the order of $10^4$-fold. Some gH–gL complexes therefore resisted antibody binding. They may be packed closely on the virion surface, making some difficult to bind to, or some may take on a form less susceptible to the mAbs that we derived. If membrane fusion requires only a few gH–gL complexes, minor, non-neutralized populations will have large functional effects. Also, gH–gL-specific mAbs may bind poorly in acidic endosomes or may require a very high binding energy to compete with an energetically favourable conformational change in gH–gL at low pH. As neutralizing mAbs were rare, the natural gH–gL-specific response is unlikely to suffice for neutralization. However, it may be possible to boost this response by vaccination. Whatever the limitations on gH–gL-directed mAbs, such vaccination probably represents the best current prospect for neutralizing gammaherpesviruses in vivo.

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