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Escape of Mutant Double-Stranded DNA Virus from Innate Immune Control

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Summary

As innate immune system components, natural killer (NK) cells respond rapidly to infections and effectively control replication of pathogens, including murine cytomegalovirus (MCMV), a double-stranded DNA β-herpesvirus. In the absence of NK cell control, MCMV infection results in early mortality due to uncontrolled viral replication. However, here we show that even in the face of initial NK cell control, there is late recrudescence of disease and mortality in immunodeficient mice due to the outgrowth of MCMV mutants that escape recognition by innate NK cells. These data suggest that viral infections in certain clinical settings also may be due to viral escape from innate immunity.

Introduction

The innate immune system effectively controls pathogens, such as viruses, during the initial phases of the host immune response (Janeway, 2001). This control is crucial to host survival, since adaptive specific immunity becomes manifest only after a lag period of a week or more during which there is clonal selection and expansion of antigen-specific T and B cell clones. In the absence of innate immune components, pathogen replication may be so rapid that infection overwhelms the host, resulting in death within the first few days of disease (Brown et al., 2001; Salazar-Mather et al., 2002). On the other hand, the absence of adaptive immunity leads to susceptibility to infection and inability to clear the pathogens as illustrated by immunocompromised human patients (Fauci and Lane, 2001; Fishman and Rubin, 1998; Schwarz et al., 1999). However, the nature of the persistent pathogens in immunodeficiency is not fully understood.

Infection of mice with murine cytomegalovirus (MCMV) has been used as a model to examine host-pathogen relationships, particularly with respect to immune responses (Mocarski, 2002; Tortorella et al., 2000; Webb et al., 2002). MCMV is a β-herpesvirus that, like other large double-stranded DNA viruses, has evolved a genome containing open reading frames (ORFs) that interact with the host immune system. These ORFs provide compelling insight into the interaction of MCMV infection with the mouse immune response. For example, the MCMV genome contains several ORFs that effectively downregulate MHC class I expression, permitting escape from $CD8^+$ cytotoxic T lymphocyte (CTL) recognition of infected cells (reviewed in Orange et al., 2002; Reddehase, 2002; Tortorella et al., 2000). However, evasion strategies targeting specific immunity may enhance innate immune responses, such as those from natural killer (NK) cells that demonstrate enhanced killing of targets that lack MHC class I expression, as predicted by the "missing-self" hypothesis (Ljunggren and Karre, 1990). Countering this host response, MCMV contains an ORF (*m144*) that appears to inhibit NK cells apparently through an as yet undefined inhibitory NK cell receptor, analogous to human CMV ORFs that encode ligands for NK cell inhibitory receptors (Farrell et al., 1997; Reyburn et al., 1997; Tortorella et al., 2000). Thus, the complex interplay between the virus and the host usually reflects long-standing co-evolution of the virus with the host immune system rather than during the context of an ongoing viral infection.

Studies of MCMV infection have also highlighted the intricate relationship between the adaptive and innate arms of the immune system. There is clearly a role for the protective effects of acquired specific immune responses, primarily from $CD8^+$ CTLs (Reddehase et al., 1987). However, mice with deficiencies in innate immune function succumb to MCMV infection before acquired immunity becomes manifest (reviewed in Biron, 1999). A major component of innate immunity, the NK cell, provides vital host innate immune defense against MCMV by killing infected cells and producing cytokines such as IFN-γ (Biron et al., 1999; Bukowski et al., 1983).

Specific NK cell control of MCMV is genetically determined by *Cmv1*, an autosomal dominant locus, outside the MHC, that renders C57BL/6 (B6) mice resistant to MCMV whereas BALB/c mice are susceptible (Scalzo et al., 1990). *Cmv1* encodes the Ly49H activation receptor expressed on about 50% of NK cells in B6 mice (Brown et al., 2001; Lee et al., 2001). Ly49H specifically recognizes the MCMV-encoded ORF product m157, a glycophosphatidylinositol (GPI)-anchored protein, on virally infected cells, and triggers NK cell-mediated cytotoxicity and cytokine production (Arase et al., 2002; Smith et al., 2002). Interestingly, mice with intact adaptive immune systems but genetically lacking *Ly49h*, such as BALB/c ($Cmv1^s$ allele), can survive infection with low

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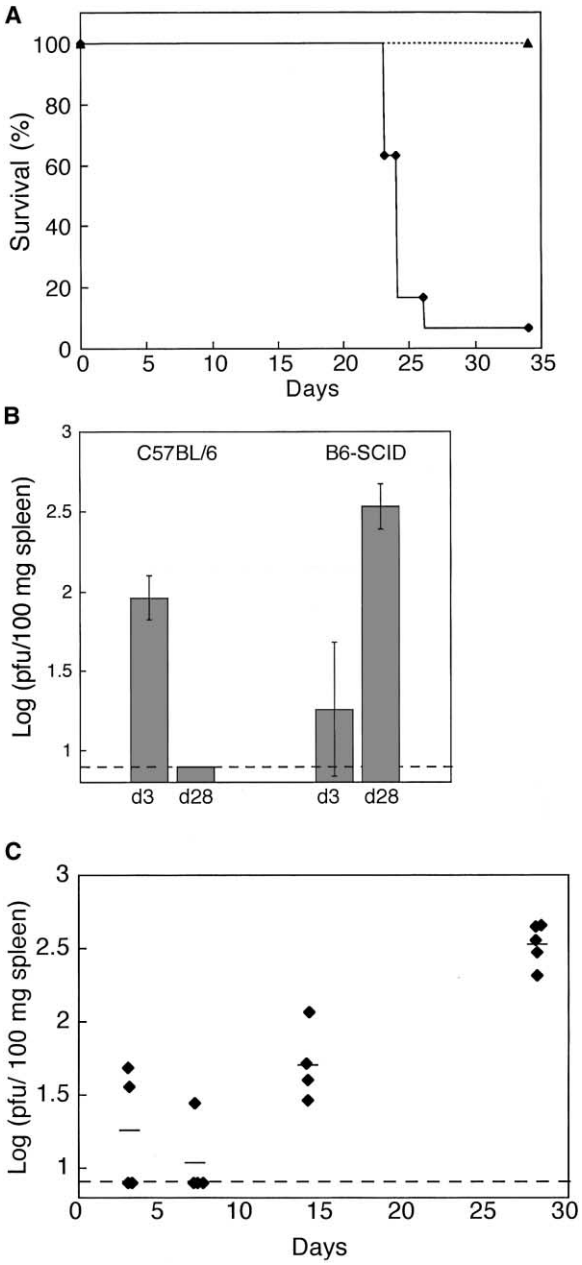

Figure 1. Survival and Splenic Titers in B6-RAG1$^{-/-}$ and B6-SCID Mice during MCMV Infection

(A) Survival curve for 30 B6-RAG1$^{-/-}$ mice infected i.p. with 2.5 $\times$ 10^4 pfu wt MCMV (Smith strain) per mouse (solid line). At this and higher doses, all wt B6 mice survive (Brown et al., 2001) as illustrated by the survival curve of six B6 mice infected i.p. with 5 $\times$ 10^4 pfu MCMV (Smith strain) per mouse (dotted line) from a separate experiment shown for comparison.

(B) Splenic titers at day 3 (d3) p.i. and day 28 (d28) p.i. in groups of four to five wt B6 or B6-SCID mice infected with 2 $\times$ 10^4 pfu/mouse. The horizontal dashed line represents the level of detection of the assay. For mice with titers below the level of detection, the minimum number of detectable pfus was used to determine the mean. The differences in splenic titers at d3 and d28 were statistically significant in both B6 ($p = 0.0007$) and B6-SCID mice ($p = 0.007$).

(C) Splenic titers at days 3, 7, 14, and 28 p.i. in groups of four to five C57BL/6-SCID mice infected with 2 $\times$ 10^4 pfu/mouse. Mean viral titers for each group are depicted as horizontal bars. (In [B] and [C], viral titers were determined in mice surviving at days indicated. Titers may have been significantly higher in mice that died prior to d28, and are therefore likely to be an underestimate of viral titers associated with death itself.)

doses of MCMV but are susceptible to challenges with higher inoculi of MCMV as evidenced by markedly elevated splenic viral titers, already evident by day 3 postinfection (p.i.), and decreased survival with deaths occurring early around day 6–7 p.i. (Scalzo et al., 1990). By contrast, Ly49H$^+$ (*Cmv1^r*) strains, such as B6 or BALB/c mice reconstituted with *Ly49h* by transgenesis, have low viral titers and survive infection with similar inoculums of MCMV (Brown et al., 2001; Lee et al., 2001, 2003; Scalzo et al., 1990). Taken together with immunological analysis (Brown et al., 2001; Daniels et al., 2001), these data demonstrate that the Ly49H NK cell activation receptor mediates acute innate immune control of MCMV and is responsible for the increased resistance of B6 mice to MCMV.

In the current studies, however, we demonstrate that innate NK cell control of MCMV infection in mice on the B6 background is inadequate for long-term survival if the host lacks adaptive immunity. After a single inoculum, there is recrudescence of disease due to MCMV mutants that escape NK cell control rather than to reemergence of the original virus. These studies have significant implications for the clinical setting as the data suggest that pathogens that cause severe disease in immunocompromised patients may represent mutants escaping innate immunity.

Results

Late Lethality in MCMV-Infected Immunodeficient Mice

Despite acute innate immune control, B6 mice lacking an adaptive immune system, i.e., immunodeficient B6-SCID or B6-RAG1$^{-/-}$ mice, experienced late deaths occurring 3 to 5 weeks p.i. (Figure 1A). Similar results were observed in several other cohorts of B6-RAG1$^{-/-}$ and B6-SCID mice infected with doses of MCMV as low as 2 $\times$ 10^4 pfu, inoculi significantly less than the LD_{50} of MCMV in wt B6 mice (2.5 $\times$ 10^5 pfu, data not shown). These results correlate well with a previous report of late deaths observed in another immunodeficient mouse (B6.RAG2$^{-/-}$) infected with MCMV (Riera et al., 2000) and contrast sharply with the much earlier deaths (day 6–7 p.i.) in mice on a genetically susceptible background such as BALB/c (including CB.17-SCID), or in otherwise resistant B6 mice with genetic or induced defects in innate immunity or NK cells (Brown et al., 2001; Bukowski et al., 1983; Salazar-Mather et al., 2002; Scalzo et al., 1990).

Whereas wt B6 mice were able to control MCMV infection with undetectable splenic titers at day 28 p.i., surviving B6-SCID mice consistently had higher titers at day 28 than at day 3 p.i. (Figure 1B; data not shown). The lower initial (day 3 p.i.) titers in B6-SCID as compared to B6 spleens may be partially explained by the proportionally greater fraction of NK cells in the B6-SCID spleen (25% versus 2.5% in wt mice). The splenic titers in B6-SCID mice decreased further from day 3 to day 7 p.i.

However, the titers then rebounded to much higher levels (Figure 1C), indicating that the immunodeficient mice were initially able to control the MCMV infection but at later time points (day 28 p.i.) the virus escaped this control (Figure 1A and 1C).

The recrudescence of disease in immunodeficient mice but not in wt mice (Figure 1) suggested that the re-emergence of MCMV is due to the absence of adaptive immune cells. Indeed, previous studies have documented the role of adaptive immunity in controlling MCMV infections in immunocompetent mice (Reddehase, 2002). The transfer of splenocytes from MCMV-challenged immunocompetent mice into MCMV-infected B6-SCID mice completely prevented elevation of late splenic viral titers (data not shown). Therefore, although Ly49H$^+$ NK cells provide effective early control of MCMV (Figure 1, Brown et al., 2001), an adaptive immune response is necessary to prevent the re-emergence of MCMV late during infection, indicating that early innate immunity alone is insufficient for long-term control of MCMV infection.

MCMV Isolates from Immunodeficient Mice

The re-emergence of MCMV late during infection in B6-SCID mice could represent the reactivation from latency of MCMV representing the original inoculum, the selection of MCMV escape mutants, or immunosuppression of innate immunity through constant stimulation from persistent occult infection, analogous to T cell exhaustion in viral infections (Zinkernagel et al., 1999). To test these hypotheses, we isolated plaque-purified MCMV from spleen homogenates of surviving B6-SCID mice at day 28 p.i. We then used these new isolates, termed SCID-MCMV, to infect B6-SCID mice. Infection with a SCID-MCMV isolate (A1.10) resulted in marked mortality in B6-SCID mice by day 7, indicating ineffective innate control (Figure 2A). Similarly there was 70%–100% mortality in infections with 3 separate SCID-MCMV isolates in wt B6 mice compared to 100% survival at 28 days with wt virus infection (Figure 2B). Similar results were observed in a second independent experiment (data not shown). The LD_{50} of SCID-MCMV isolate A1.10 was 3×10^4 pfu, about one-tenth the LD_{50} of wt MCMV, passaged an equivalent number of times in vivo (data not shown). Infection with these isolates in B6 mice resulted in significantly higher day 3 splenic titers than infection with the same dose of wt MCMV (Figure 2C). More modest differences (3- to 14-fold) were seen between day 3 hepatic titers in mice infected with wt MCMV and SCID-MCMV isolates (Figure 2D) consistent with previous observations that the *Cmv1^r* MCMV resistance locus (*Ly49h*) is characterized primarily by decreased splenic titers and increased survival with significantly less effect on hepatic titers (Brown et al., 2001). These data strongly suggest that the elevated titers observed late in the infection of SCID mice (Figure 1C) result from changes in the virus and not from exhaustion of host innate immunity because the phenotype of uncontrolled viral replication and death could be recapitulated with the SCID-MCMV isolates early in the course of infection when B6-SCID and wt B6 mice are normally resistant.

Lack of Ly49H NK Cell Stimulation by SCID-MCMV Isolates

Instead of re-emergence of the original virus, the data suggested that MCMV isolated during recrudescence of infection in B6-SCID mice were mutants that had escaped from NK cell control because the SCID-MCMV isolates were unrestrained when reintroduced into immunocompetent wt B6 mice. This phenotype (mortality, time to death, elevated viral titers) resembled that of infections with wt MCMV in NK cell depleted or Ly49H-deficient mice (Brown et al., 2001; Bukowski et al., 1983; Scalzo et al., 1990). Based on this evidence, we hypothesized that the SCID-MCMV isolates had escaped NK cell control by specifically affecting Ly49H stimulation of NK cells. To evaluate this possibility, we took advantage of previous observations (Smith et al., 2002) recapitulated here (Figure 3A) that in vitro co-incubation of splenocytes with wt MCMV-infected IC-21 macrophages results in downregulation of Ly49H expression and enhanced selective stimulation of IFN-γ production from Ly49H$^+$ NK cells due to direct activation through Ly49H itself. In contrast, co-incubation of C57BL/6-RAG1$^{-/-}$ splenocytes with IC-21 macrophages infected with SCID-MCMV isolates did not stimulate these responses in Ly49H$^+$ NK cells (Figure 3A). In addition, in vivo analysis of B6 mice infected with wt MCMV revealed selective expansion of the Ly49H$^+$ NK cells as indicated by the increased percentage of splenic Ly49H$^+$ NK cells following infection (Figure 3B). Previous studies have shown that this expansion is due to selective Ly49H stimulation and proliferation of Ly49H$^+$ NK cells (Dokun et al., 2001). On the contrary, when B6 mice were infected with similar inoculi of SCID-MCMV isolates, there was no shift in the proportion of NK cells that were Ly49H$^+$. Similar results were observed when B6 mice were infected with lower doses of MCMV (data not shown). Interestingly, early (36 hours p.i.) Ly49H-independent "non-specific" in vivo stimulation of NK cells (proliferation and IFN-γ production) (Dokun et al., 2001) during infection with SCID-MCMV isolates was not attenuated, as compared to infection with wt MCMV (data not shown). Taken together, these data demonstrate that the SCID-MCMV isolates manifest selective abnormalities in the specific in vitro and in vivo stimulation of Ly49H$^+$ NK cells.

m157 Mutations in SCID-MCMV Isolates

Specific stimulation of Ly49H$^+$ NK cells requires Ly49H recognition of MCMV-encoded m157 (Arase et al., 2002; Smith et al., 2002). We therefore hypothesized that there may be mutations in *m157* in the SCID-MCMV isolates facilitating escape from innate immune control. Indeed, the vast majority of splenic SCID-MCMV isolates in eleven mice from three separate experiments were found to have mutations in *m157* (Table 1, each experiment designated by a different letter, A, B, or C). In sequencing the 993 nucleotides (nt) comprising the *m157* ORF from 64 independent splenic isolates, we found mutations in 61 ($>$95%). The most common mutation was a 13 nt deletion in the predicted membrane proximal hydrophobic tail of m157 that would preclude GPI linkage. Other deletions or substitutions change the frame or result in premature stop codons. Indeed, 56 of

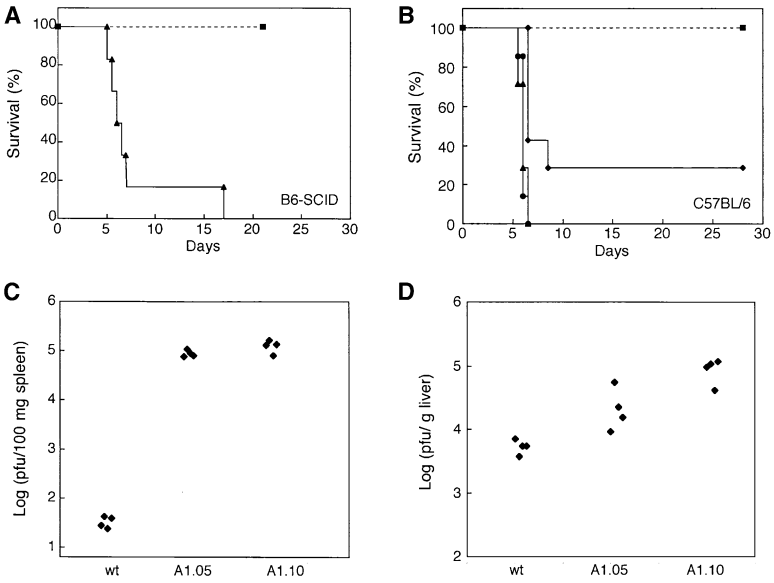

Figure 2. Increased Mortality and Splenic and Hepatic Titers in B6-SCID and B6 Mice Infected with SCID-MCMV Isolates

(A) Survival curves of groups of 6 B6-SCID mice infected i.p. with (5×10^4 pfu/mouse) wt MCMV (squares) or SCID-MCMV A1.10 isolate (triangles).

(B) Survival curves of groups of seven B6 mice infected i.p. (6×10^4 pfu/mouse) with wt MCMV (squares) or SCID-MCMV isolates including A1.05 (circles) and A1.10 (triangles), both of which have a 3′ thirteen nucleotide deletion, and A1.04 (diamonds), which has a 5′ deletion. Results are representative of two independent experiments. A wt MCMV stock that had been passed an additional time through BALB/c mice so that it had the same number of in vivo passages through BALB/c mice as the SCID-MCMV isolates had an identical LD_{50} as the parental wt stock (2.5×10^5 PFU), and a group of B6 mice infected with this wt MCMV stock (6×10^4 pfu/mouse) all survived (data not shown) similar to mice infected with the parental wt stock.

(C) Splenic titers at day 3 p.i. in groups of four B6 mice infected with 2×10^4 pfu/mouse of either wt MCMV or two SCID-MCMV isolates (A1.05 and A1.10). The d3 splenic titers in B6 mice infected with the SCID-MCMV isolates were statistically distinct from titers in mice infected with wt MCMV ($p = 0.0000001$ for A1.05 and $p = 0.00000002$ for A1.10).

(D) Hepatic titers at day 3 p.i. in the same groups of four B6 mice infected with 2×10^4 pfu/mouse of either wt MCMV or two SCID-MCMV isolates (A1.05 and A1.10). The differences in hepatic titers in B6 mice infected with wt MCMV or SCID-MCMV isolates were statistically significant ($p = 0.03$ for A1.05 and $p = 0.0003$ for A1.10).

the 61 mutations identified are predicted to disrupt the expression and retention of the full-length m157 product. In striking contrast, sequencing of two adjacent ORFs that belong to the same *m145* family in the MCMV genome as *m157* revealed no mutations in *m158* (1071 nt) in 13 isolates or in *m159* (1197 nt) in 12 isolates all of which contained *m157* mutations. While there may be additional mutations in other MCMV ORFs that were not detected in our analysis of these three genes, these results clearly demonstrate that *m157* mutants were selected during MCMV infection in B6-SCID mice.

We also found elevated hepatic titers on day 28 p.i. in B6-SCID mice (data not shown). Sequencing demonstrated *m157* mutations in 19 out of 20 hepatic SCID-MCMV isolates (Table 2) closely correlating with the *m157* mutations found in splenic SCID-MCMV isolates from the same mice (Table 1). These results demonstrate that mutant MCMV is disseminated and that the spleen is not a privileged site to observe *m157* mutations.

The experiments presented in Tables 1 and 2 were done with salivary gland-passaged MCMV stocks. No *m157* mutations were detected in nine clones of the original salivary gland MCMV stock previously passaged in BALB/c mice (data not shown). Although these experiments demonstrated that *m157* mutants were selected during the course of infection in B6-SCID mice, no conclusion could be drawn about whether the mutations occurred during the course of infection or represented the outgrowth of previously existing rare viral subpopulations in the salivary gland MCMV stock.

To more directly address this question, we repeated the experiment with a plaque-purified wt MCMV clone with a verified normal *m157* sequence (Table 3). When tissue culture preparations of this plaque-purified MCMV clone were used to directly infect B6-SCID mice, elevated splenic titers on day 28 were again observed (data not shown). Sequencing of *m157* in 28 splenic isolates from four B6-SCID mice revealed mutations in all the isolates (Table 3). Substitutions or deletions resulting in premature stop codons in *m157* were found in 25 of 28 mutated isolates. Interestingly, each mouse had an independent dominant *m157* mutation that was not seen in the other mice. These results strongly support the hypothesis that MCMV is mutating during the course of infection in the mice and being selected under pressure from NK cells.

Whereas other mutations may also be present in the SCID-MCMV isolates and contribute to their increased virulence, deliberate mutation of *m157* (Δm157) recapitulated the SCID-MCMV phenotype of elevated viral titers (Figure 3C) and inability to activate $Ly49H^+$ NK cells (Figure 3A). The phenotype of Δm157 was restored to that of wt MCMV by intentional re-introduction of *m157*. Furthermore, studies of Δm157 support the thesis that *m157* alone is involved in the genetic resistance of B6 mice to MCMV via Ly49H (Bubic et al, submitted). Thus, the *m157* mutations are sufficient to explain the escape virus phenotype of the SCID-MCMV isolates.

Discussion

Over eons of interacting with their hosts, viruses have evolved a number of strategies to evade immune responses (Tortorella et al., 2000). Whereas double-stranded DNA viruses, such as the herpesviruses, have developed ORFs that neutralize host immune responses, virus mutants that escape immunity during the course of an infection have been described primarily for RNA viruses, and in the context of evasion from adaptive not innate immunity (Ciurea et al., 2001; Erickson et al.,

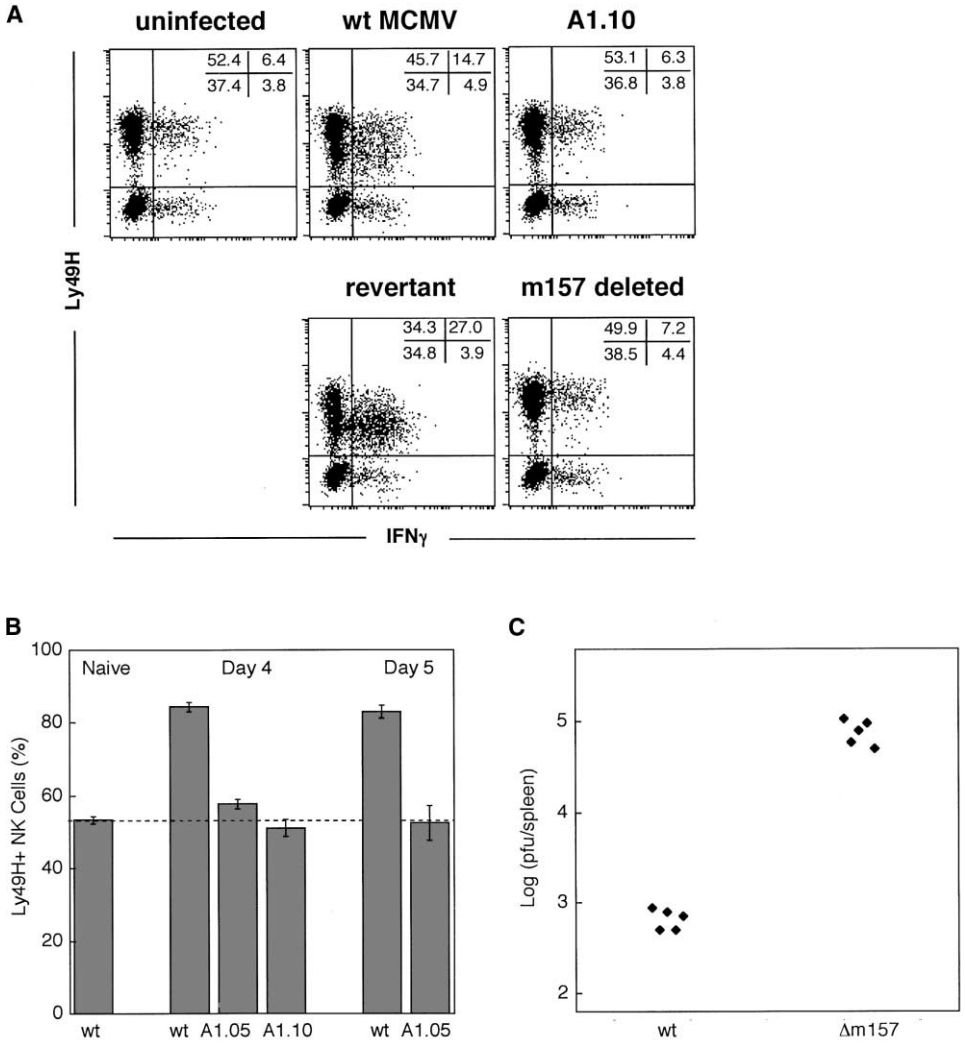

Figure 3. Disruption of the Ly49H-m157 Interaction during Infection with SCID-MCMV Isolates

(A) B6-RAG1$^{-/-}$ splenocytes were cocultured for 9 hr with noninfected IC-21 cells or IC-21 cells previously infected with wt MCMV, SCID-MCMV isolate (A1.10), Δm157 (deliberate mutation), or revertant MCMV. The cells were then stained for NK1.1, CD3, and Ly49H and fixed, permeabilized, and stained for intracellular IFN-γ. The results shown are gated on NK1.1$^+$ CD3$^-$ lymphocytes.

(B) Splenic NK cells were harvested from B6 mice infected with 5×10^4 pfu/mouse of wt MCMV or SCID-MCMV isolates (A1.05, A1.10). The cells were stained for NK1.1, CD3, and Ly49H. The percentage of NK cells (NK1.1$^+$ CD3$^-$) that were Ly49H$^+$ was determined by FACS analysis. The differences between the % Ly49H$^+$ NK cells in naive B6 mice (indicated by dashed line) and mice d4 or d5 p.i. with wt MCMV were statistically significant ($p < 0.05$) while the differences between naive mice and mice infected with the SCID-MCMV isolates were not. (Data represent the average of groups of three to four mice).

(C) Splenic titers at day 3 p.i. in groups of five B6 mice infected with 5×10^5 pfu/mouse of either tissue-cultured propagated wt or Δm157 (deliberate mutation) MCMV. The differences between the d3 splenic titers in B6 mice infected with wt or Δm157 MCMV were statistically significant ($p = 0.00000001$).

2001; Goulder et al., 2001; Hunziker et al., 2003; Schrag et al., 1999). Here we report that in the absence of an adaptive immune response, Ly49H$^+$ NK cells exert enough selective pressure to permit the specific outgrowth of MCMV escape mutants with alterations in m157 sufficient to disrupt its interaction with Ly49H on NK cells and lead to increased splenic titers and mortality. To our knowledge, this is the first description of the in vivo emergence, during the course of a single infection, of escape mutants of a double-stranded DNA virus driven by innate immune selective pressure.

Most prior studies of mutant viruses and host defense have dealt with RNA viruses that have significant propensity towards the development of "quasispecies" due to the inherent infidelity of RNA polymerase (DeFilippis and Villareal, 2001). Our results demonstrate that similar phenomena may occur during the course of infection from a single inoculum of a double-stranded DNA virus even though DNA viruses are thought to be less prone to mutations due to higher fidelity of DNA replication (DeFilippis and Villareal, 2001). Our initial experiments revealed the isolation of a number of identical mutants in different mice that could result from regions in *m157* with a higher predisposition to mutation. However, these

Table 1. Mutations in *m157* in Splenic SCID-MCMV Isolates from B6-SCID Mice Infected with Salivary Gland-Passaged MCMV

Mouse	Frequency	Mutation	Comment
A1	1/5	305-323 deletion	frameshift w/premature stop at 431-433
	4/5	961-973 deletion	disrupt predicted hydrophobic tail
A2	1/4	G881A	premature stop (TAG)
	3/4	961-973 deletion	disrupt predicted hydrophobic tail
A3	1/2	G881A	premature stop (TAG)
	1/2	961-973 deletion	disrupt predicted hydrophobic tail
B1	1/10	no mutation	
	1/10	647-648 deletion	frameshift w/premature stop at 699-701
	2/10	G881A	premature stop (TAG)
	6/10	961-973 deletion	disrupt predicted hydrophobic tail
B2	1/8	no mutation	
	2/8	G509A	gly → asp
	1/8	820-918 deletion	
	1/8	addition of A at 789	premature stop (TAA)
	2/8	G881A	premature stop (TAG)
	1/8	961-973 deletion	disrupt predicted hydrophobic tail
B3	1/10	no mutation	
	5/10	647-648 deletion	frameshift w/premature stop at 699-701
	4/10	961-973 deletion	disrupt predicted hydrophobic tail
C1	1/5	412-414 deletion	deletion of Thr
	1/5	C485A	premature stop (TAA)
	1/5	G881A	premature stop (TAG)
	2/5	961-973 deletion	disrupt predicted hydrophobic tail
C2	3/5	G881A	premature stop (TAG)
	2/5	961-973 deletion	disrupt predicted hydrophobic tail
C3	4/5	G881A	premature stop (TAG)
	1/5	961-973 deletion	disrupt predicted hydrophobic tail
C4	5/5	961-973 deletion	disrupt predicted hydrophobic tail
C5	1/5	G398T	cys → tyr
	1/5	G418T	asp → tyr
	1/5	647-648 deletion	frameshift w/premature stop at 699-701
	1/5	addition of A at 789	premature stop (TAA)
	1/5	G881A	premature stop (TAG)

Eleven B6-SCID mice were infected with salivary gland-passaged Smith strain MCMV (2×10^4 pfu/mouse) in three separate experiments (denoted by A, B, and C). Homogenates of spleens harvested on day 28 p.i. were used to infect monolayers of NIH-3T12 cells. Five days later, viral plaques were isolated. From each isolate, viral genomic DNA was harvested, and *m157* was PCR amplified for sequencing. Similar analysis of SCID-MCMV isolates revealed no mutations in *m158* (13 isolates) or in *m159* (12 isolates) even though the same isolates possessed *m157* mutations (not shown). No *m157* mutations were found in 9 wt MCMV clones isolated from the salivary gland stock, previously passaged in BALB/c mice (not shown).

experiments were conducted with an in vivo passaged salivary gland MCMV preparation, raising the possibility that the mutants may have been present at low frequencies in the preparation. When we used a newly cloned MCMV preparation with an intact *m157* sequence, we found clustering of distinct *m157* mutations in individual mice, which were different from mutations found in mice infected with the salivary gland inoculum. These results support the possibility that several of the commonly identified mutants in the initial experiments did exist as rare subpopulations within the salivary gland stock and were selected during the infection. More importantly, the data with the tissue culture passaged inoculum strongly suggest that MCMV is mutating during the course of infection.

Regardless of whether the mutant viruses were preexistent in the inoculum or developed de novo during infection, it is clear that viruses emerging later during the course of infection had been selected and did not simply represent re-activation from latency of the original virus since the SCID-MCMV isolates had differences in *m157* sequence and biological behavior compared to the original inoculum. Perhaps these mutations occurred during ongoing viral replication in an immune privileged site, such as the salivary gland that manifests viral persistence despite a vigorous immune response (Cavanaugh et al., 2003). Seeding of the periphery with mutant viruses from the salivary gland may have led to viremia and NK cell selection in the spleen where the Ly49H-mediated response is most evident (Scalzo et al., 1992; Tay and Welsh, 1997).

The striking increased frequency of mutations in *m157* in SCID-MCMV isolates following infection with either salivary gland-passaged MCMV or plaque-purified, tissue-cultured MCMV demonstrates that innate immune control by NK cells in the absence of an adaptive immune response resulted in the selective outgrowth of viruses that have abnormalities in *m157*. The increased frequency of *m157* mutations sharply contrasted with the absence of mutations found in two other adjacent *m145* family members (*m158* and *m159*) even though all three of these ORFs are dispensable for in vitro viral propagation (Thale et al., 1995). Detailed analysis of the complete genomic sequence of the SCID-MCMV isolates may reveal additional mutations that potentially contribute to their biological properties. However, the majority of *m157* mutations that were identified would prevent expression and retention of m157 on infected cells, and a deliberate mutation in *m157* alone recapitulated the phenotype observed with the SCID-MCMV iso-

Table 2. Mutations in *m157* in Hepatic SCID-MCMV Isolates from B6-SCID Mice Infected with Salivary Gland-Passaged MCMV

Mouse	Frequency	Mutation	Comment
C1	1/5	no mutation	
	1/5	647-648 deletion	frameshift w/premature stop at 699-701
	3/5	961-973 deletion	disrupt predicted hydrophobic tail
C2	3/5	G881A	premature stop (TAG)
	2/5	961-973 deletion	disrupt predicted hydrophobic tail
C3	1/5	addition of A at 789	premature stop (TAA)
	3/5	G881A	premature stop (TAG)
	1/5	961-973 deletion	disrupt predicted hydrophobic tail
C4	1/5	G475T	premature stop (TGA)
	1/5	G881A	premature stop (TAG)
	3/5	961-973 deletion	disrupt predicted hydrophobic tail

SCID-MCMV isolates were obtained from liver homogenates of four of the five B6-SCID mice in experiment C described in Table 1, and *m157* was sequenced.

lates, indicating that the mutations in *m157* are sufficient to account for the increased virulence observed with the SCID-MCMV isolates.

Why then does MCMV have *m157* in the first place, and why was it not deleted previously? There are several points worth considering. First, as opposed to many other viruses, a prominent feature of the herpesvirus life cycle is the establishment of latency (Knipe et al., 2001); *m157* may play a role in this regard since it provides the virus with a mechanism to limit acute host demise. Perhaps it is advantageous to the virus that the host survive to develop a chronic latent infectious state. Over the lifetime of the infected host, re-activation will lead to low-level infections that can then be spread to many other acquaintances. By contrast, an acute overwhelming infection provides only a very short period when the host is well enough to spread the virus. Paradoxically then, immune stimulation by m157 may represent a viral immune modulating strategy that is beneficial from the virus standpoint. Second, m157 may play another role in immune evasion. Previously, m157 was shown to bind an NK cell inhibitory receptor (Ly49I) in the 129 strain but not C57BL/6 mice (Arase et al., 2002). However, the relevance of this interaction is unclear due to the limited expression of Ly49I on only 10% of NK cells in 129 strain mice. Yet it is possible that m157 may bind a larger number of NK cell inhibitory receptor-positive cells in other strains or outbred mice, or it may have other immune evasion functions in an analogous manner to a related molecule (m152) involved in downregulation of MHC class I molecules and NKG2D ligands (Krmpotic et al., 2002; Smith et al., 2002; Tortorella et al., 2000). Third, there is only limited genomic information on MCMV, and this is confined to two related laboratory strains. Recent studies have indicated that mutations in *m157* can also be identified in MCMV isolates from wild mice but the genotype of their hosts with respect to *Ly49h* was not determined (Voigt et al., 2003). *m157* mutations were also identified after sequential salivary gland passage of K181 strain MCMV in congenic Ly49H$^+$ mice, indicating that mutant viruses were resident in salivary glands. However, selection of *m157* mutants became evident only after multiple rounds of sequential passage in otherwise immunocompetent mice (Voigt et al., 2003). Similarly, the studies reported here indicate that mutant viruses were not detectable after a single inoculum in the presence of adaptive immunity, presumably due to effective suppression by CTLs and perhaps due to other effects of *m157* in modulating host immune responses. Further analysis is clearly required to address these issues.

Selection pressure by the immune system has been previously reported to influence the outcome of infectious challenges. Several examples include the mutation of antigenic epitopes for virus-specific immunoglobulin or TCR (Ciurea et al., 2001; Lopez-Bueno et al., 2003). However, in almost all cases reported thus far, the viruses escaped adaptive immune control. Here we show directly that a naturally occurring, mono-specific innate immune control mechanism results in readily detectable viral mutations, indicating that innate immunity is insufficient for long-term infection control.

In contrast to the plasticity of antigen specificities that are available in unmanipulated adaptive immune responses, innate immune control mechanisms are much more limited. Innate immune cells have germline-

Table 3. Mutations in *m157* in Splenic SCID-MCMV Isolates from B6-SCID Mice Infected with Plaque-Purified, Tissue-Cultured Propagated MCMV

Mouse	Frequency	Mutation	Comment
D1	5/7	extra A at 305	frameshift with stop at 315-317 TAA
	2/7	G644A	TGT => TAT (cys => tyr)
D2	8/8	G538T	premature stop (TAA)
D3	1/8	T179G	ATT => ATG (ile => met)
	7/8	T840A	premature stop (TAA)
D4	5/5	deletion of A at 304	premature stop at 311 (TAA)

Smith strain MCMV was plaque-purified from the original salivary gland stock and sequenced to verify that *m157* was intact. After only tissue culture amplification, this MCMV preparation was used to infect four B6-SCID mice (D1 and D2 at 5×10^4 pfu/mouse and D3 and D4 at 5×10^5 pfu/mouse). Individual viral plaques were isolated from splenic homogenates, and *m157* was sequenced as described in Table 1.

encoded receptors, such as Ly49H and those involved in pattern recognition, such as the Toll-like receptors. These receptors are often promiscuous in ligand binding but the repertoire is quite limited. There has been no evidence thus far for somatic mutations of these receptors to increase their repertoire for a larger panel of ligands or to deal with mutating pathogens. Thus, more detailed analysis of infectious challenges may reveal mutations of viruses or micro-organisms that escape these receptors of innate immunity, but given the multilayered immune control normally suppressing the outgrowth of escape mutants, these mutations may be evident primarily when other arms of the immune system are disabled.

Taken in a broader context, therefore, our findings have significant implications in the clinical setting. In immunodeficient humans, such as AIDS or immunosuppressed organ transplant patients, the absence of competent adaptive immunity is associated with the development of severe viral illnesses, such as CMV viremia, meningitis, pneumonia, and retinitis, similar to the widespread disease in our infected SCID and RAG$^{-/-}$ mice (Fauci and Lane, 2001; Fishman and Rubin, 1998). Severe or recurrent viral infections have also been reported in patients with selective genetic defects in adaptive immunity but with intact functional NK cell populations, such as severe combined immunodeficiency patients with defects in RAG1/2 (T- NK+ SCID) (Schwarz et al., 1999). Interestingly, in immunosuppressed transplant patients, CMV disease typically occurs relatively late (more than a month), rather than acutely, after lung transplants from CMV^{+} donors, a time course again reminiscent of our studies (Fishman and Rubin, 1998). Furthermore, in uncontrolled infections in immunocompromised patients, the nature of the viruses has not been fully characterized. Here we show that mutated forms and not the original virus emerge during the course of infection. We therefore predict that certain viral infections in immunodeficient patients may be due to viruses escaping innate immunity and that genomic analysis of such clinical isolates may provide valuable clues to the viral products that interact with various components of the host innate immune system.

Experimental Procedures

Mice

C57BL/6 (B6), C57BL/6-SCID (B6-SCID), and C57BL/6-RAG1$^{-/-}$ (B6-RAG) mice were obtained from the Jackson Laboratory (Bar Harbor, ME). Mice were maintained under specific pathogen-free conditions and used between 8–14 weeks of age. All experiments were conducted in accordance with institutional guidelines for animal care and use.

Virus and Infection of Mice

Smith strain MCMV was a generous gift of H. Virgin (Washington University, St Louis, MO). A salivary gland stock of MCMV was prepared from BALB/c mice that had been intraperitoneally (i.p.) injected with 1×10^6 pfu tissue-culture propagated MCMV, and the titer was determined via standard plaque assay using permissive NIH 3T12 fibroblasts (American Type Culture Collection, ATCC, Manassas, VA) (Brown et al., 2001). For in vivo experiments, mice were injected i.p. with 2×10^4 to 1×10^5 pfu/mouse from a salivary gland MCMV stock with a titer of 5×10^6 pfu/ml. Alternatively, some experiments were performed with tissue-culture propagated, plaque-purified wt MCMV (with verified m157 sequence), as indicated.

Directed Mutation of m157 in MCMV

Directed mutagenesis of the *m157* ORF (nt 215898-216884 encoded on the complementary strand) was performed on the bacterial artificial chromosome (BAC) pSM3fr containing the wt MCMV (Wagner et al., 1999) following a previously described method (Wagner et al., 2002). The BAC-derived MCMV is a Smith strain MCMV with genes 151 to 158 derived from the K181 MCMV strain. However, *m157* was confirmed to be identical in sequence in both Smith and K181 strains. Mutagenesis of *m157* was performed by deletion of nucleotides 216291-216884 (Δm157) via homologous recombination in *E. coli*. A revertant virus was generated by inserting the *m157* ORF and its native promoter ectopically between ORF *m16* and *m17* (between nt 15678 and 15759). A more complete description of the generation and characterization of Δm157 has been submitted as a separate manuscript (I.B. et al., unpublished data).

Plaque Assays

Viral titers were determined by standard plaque assay (Brown et al., 2001). In brief, organs from infected mice on day 3 p.i. were weighed and frozen in 1 ml aliquots of D10 media (DMEM (Gibco) with 10% calf serum (Hyclone), 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine). Spleens or livers were thawed and homogenized with Dounce homogenizers on ice. Serial dilutions of the spleen or liver lysates were then used to infect monolayers of permissive NIH-3T12 fibroblasts in triplicate on six-well plates. After a 1 hr incubation period, the cells were overlaid with overlay media (D5 media with 1% HEPES and 0.5% noble agar [Difco]). On day 3, the cells were again overlaid with overlay media. Titers were read in triplicate by two independent observers on day 4 using a light microscope with 4× magnification. Titers are presented as Log (pfu/100 mg spleen) or Log (pfu/1 gm liver).

Propagation of Mutant Virus

Individual viral plaques were isolated from monolayers of NIH-3T12 cells 5 days after inoculation with spleen or liver homogenates of B6-SCID mice harvested at day 28 p.i. The viral plaques were resuspended in 0.5 ml of media and expanded by in vitro propagation in monolayers of NIH-3T12 in six-well plates. After *m157* sequencing, nine of these mutant strains of virus (seven with the same thirteen nucleotide 3' deletion, one with a 5' deletion, and one with a point mutation from the experiment with plaque-purified MCMV in Table 3) were propagated in MCMV-sensitive BALB/c mice to prepare salivary gland preparations for in vivo experiments. In addition, tissue culture propagated preparations were generated for some experiments, as indicated.

Viral Genetic Analysis

MCMV genomic DNA was extracted using QIAmp DNA Blood minikits (Qiagen) and quantified with a spectrophotometer. MCMV ORFs were PCR amplified using TAQ polymerase (Promega) with the following primers (10 pmol/reaction) CTT GTT AGT GCC GGT GTC TGT and CAT GGT ACA CAA ACG CAG A (*m157*), TGA CCA TGT CAA GAG GTA CTG and TCT CAT CGA GTC GTG TGC CG (*m158*), and GAA AAT ATA GTT AGC ACC GTT AG and CGC TTC TAT ACA GAT AAG GGC (*m159*). PCR products were purified with QIAquick PCR purification kits (Qiagen). Sequencing reactions were prepared using ABI Big Dye Terminators v 2.0 or v 3.1 following standard ABI protocols. Sequencing was performed on a 377 DNA Sequencer (ABI Prism) as well as by the core sequencing facility at Washington University.

Splenocyte Preparation, Intracellular Staining, and Flow Cytometry

Four or five days after infection, mice were euthanized and single-cell suspensions of splenocytes were prepared using standard techniques (Dokun et al., 2001). The splenocytes were stained with PerCP-145-2C11 (CD3, Pharmingen), APC-PK136 (NK1.1, Pharmingen), PE-streptavidin (Pharmingen), biotinylated-3D10 (Ly49H, Smith et al., 2000), or biotinylated-4E4 (Ly49D, Idris et al., 1999). To block nonspecific binding of antibodies to Fc receptors, all antibod-

ies were diluted in presence of mAb 2.4G2 (Fcγ II/III receptor, ATCC). An in vitro assay to detect preferential IFN-γ production was performed as previously described (Smith et al., 2002). In brief, C57BL/6-RAG1$^{-/-}$ splenocytes were cocultured for 9 hr with uninfected IC-21 cells (ATCC) or IC-21 cells previously infected with either tissue-culture propagated wt MCMV or mutant MCMV at an MOI of 5 for 24 hr. Brefeldin A (BD Pharmingen) was added for the last 8 hr of the coincubation. Cells were fixed and permeabilized using Cytofix/Cytoperm kit (BD Pharmingen) and then stained with FITC-XMG1.2 (IFN-γ, Pharmingen). Cells were analyzed using a FACSCalibur cytometer (BD Biosciences) gating on the NK1.1^{+} CD3^{-} populations.

Statistical Analysis

Heteroscedastic two-tailed student t-test was used to determine statistically significant differences ($p < 0.05$).

Survival Curves

Mice were injected i.p. with wt MCMV or MCMV-SCID isolates at the indicated dosages and observed daily for 28 days. Moribund mice were euthanized per institutional guidelines.

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