JOURNAL OF VIROLOGY, Apr. 2006, p. 4200–4202 Vol. 80, No. 8
0022-538X/06/$08.00+0 doi:10.1128/JVI.80.8.4200–4202.2006

Murine Cytomegalovirus Interference with Antigen Presentation Contributes to the Inability of CD8 T Cells To Control Virus in the Salivary Gland

Xiuju Lu, Amelia K. Pinto, Ann M. Kelly, Kathy S. Cho, and Ann B. Hill*

Department of Molecular Microbiology and Immunology, L220, Oregon Health and Science University, 3181 SW Sam Jackson Park Road, Portland, Oregon

Received 22 September 2005/Accepted 2 February 2006

Compared to other organs, murine cytomegalovirus (MCMV) replication in the salivary gland is uniquely resistant to CD8 T-cell control. The contribution of viral genes that interfere with antigen presentation (VIPRs) to this resistance was assessed using a mutant lacking MCMV's known VIPRs. Salivary gland titers of the VIPR-deficient virus were at least 10-fold lower than those of the wild type during the persistent phase of infection; the defect was reversed by depleting CD8 T cells. Thus, VIPRs contribute to CD8 T cells' inability to control virus in the salivary gland.

Cytomegaloviruses (CMVs) are ubiquitous, species-specific members of the beta subfamily of herpesviruses. They establish asymptomatic, lifelong infection of the immunocompetent host but can cause severe or fatal infection in the immunocompromised. Experimental infection of mice with murine CMV (MCMV) is widely used to explore the relationship between this highly successful pathogen and its hosts (14). MCMV infection is characterized by three phases. Acute infection of central visceral organs is rapidly controlled but is followed by persistent infection of the salivary gland for several weeks. Finally, latent infection is established for the life of the host.

Previous studies have revealed a unique pattern of immune control of MCMV in the salivary gland (9, 10). CD8 T cells are able to eliminate replicating virus from all central organs and from salivary gland fibroblasts. However, virus replicating in salivary gland acinar epithelial cells is resistant to CD8 T-cell control, and CD4 T cells secreting gamma interferon (IFN-γ) are required to control salivary gland infection (12). Since acinar epithelial cells are responsible for the majority of virus production in the salivary gland, CD8 T-cell depletion has little or no impact on salivary gland virus titers. The reason for CD8 T cells' impotence in this site is not clear. A recent, detailed study of the immunological milieu of the MCMV-infected salivary gland showed that CD8 T cells are recruited there in large numbers and that they remain highly functional, readily lysing MCMV-infected fibroblasts and secreting IFN-γ in response to antigen directly ex vivo (1). Furthermore, the infected salivary gland contained high levels of IFN-γ, which should promote CD8 T-cell recognition. Because CD8 T cells can control virus in other sites but not in salivary gland acinar epithelial cells, it has been suggested that these cells may be peculiarly resistant to the antiviral actions of CD8 T cells (1).

We began to study the salivary gland in an attempt to understand the biological significance of MCMV's genes that interfere with antigen presentation to CD8 T cells. We have called genes with this function "VIPRs" (18) to distinguish them from other types of immunoevasins. MCMV has three VIPRs: *m4*, *m6*, and *m152* (13, 14). Together, these genes profoundly impair CD8 T-cell lysis of MCMV-infected cells in vitro (2). In vivo, *m152* was shown to have a profound impact on the ability of adoptively transferred CD8 T cells specific for a D^b-restricted epitope to control virus replication in the lungs in a bone marrow transplantation model (7). However, the impact of these genes on the course of acute infection of immunocompetent animals is more modest. While *m152* has been shown to have some CD8 T-cell-dependent impact on acute virus titers in neonatal BALB/c mice (11), no significant impact of the VIPRs was seen on acute infection in adult C57BL/6 mice (2). All herpesviruses appear to include VIPRs, suggesting that interference with CD8 T cells is important for some aspect of the herpesvirus lifestyle (15). We had originally thought that VIPRs would be necessary for herpesviruses to maintain lifelong infection of their hosts in the face of a primed immune response. However, a virus lacking *m4*, *m6*, and *m152* was unimpaired in its ability to establish latent infection in C57BL/6 mice (2). Furthermore, judged by the ongoing, active immune response, the pattern of lifelong colonization of the host was similar for wild-type and VIPR-deficient viruses in both C57BL/6 (2) and BALB/c (6) mice. Since interference with antigen presentation to CD8 T cells was not necessary for MCMV's ability to establish and maintain latent infection, we postulated that it might be necessary to enable the virus to replicate to high titers in the salivary gland in the presence of fully primed CD8 T cells.

To investigate whether MCMV's VIPRs contribute to the unique resistance of the salivary gland to CD8 T-cell control, we decided to compare infection of BALB/c mice with MCMV lacking *m4*, *m6*, and *m152* (Δm4+m6+m152) (16) and infection with its parental BAC-derived virus (MW97.01) (17). These viruses have been shown to grow equivalently in vivo in immunodepleted mice (16). The CD8 T-cell response to MCMV in BALB/c mice is dominated by two specificities, an L^d-restricted epitope from the IE1 protein (pp89) and a K^d-

* Corresponding author. Mailing address: Department of Molecular Microbiology and Immunology, L220, Oregon Health and Science University, 3181 SW Sam Jackson Park Road, Portland, OR 97239. Phone: (503) 494-0763. Fax: (503) 494-6862. E-mail: hillan@ohsu.edu.

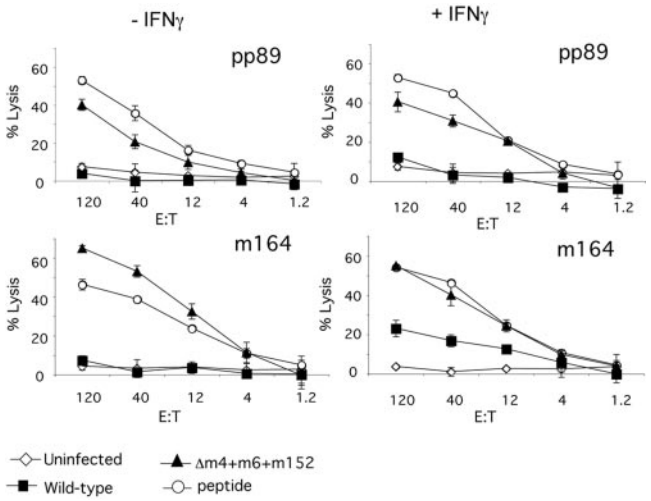

FIG. 1. Impact of MCMV's VIPRs on CTL lysis of cells infected with wild-type or Δm04+m06+m152 MCMV. Polyclonal CTL lines were treated by stimulating splenocytes with 10^{-9} M pp89 or m164 peptide and cultured for 11 days; interleukin-2 (10 U/ml) was added after day 3. BALB/c MEFs were treated with 50 U/ml IFN-γ for 24 h and then infected with the indicated virus (multiplicity of infection, 20) in the presence of phosphonoacetic acid or pulsed with peptide (10^{-6} M) before use in a Cr release assay as previously described (2). Graphs show means ± standard deviations of triplicate wells. E:T, effector-to-target cell ratio.

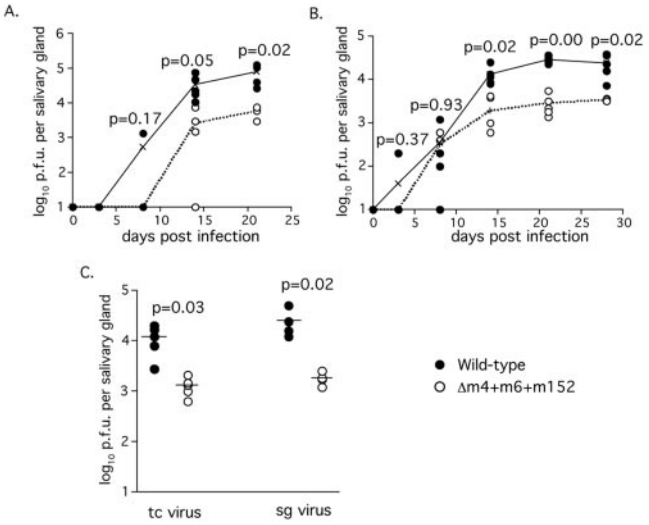

FIG. 2. Virus titers in the salivary gland after infection with wild-type or Δm4+m6+m152 MCMV. (A and B) BALB/c mice were infected intraperitoneally with 3×10^5 (A) or 5×10^6 (B) PFU of BAC-derived wild-type (MW97.01) or Δm4+m6+m152 MCMV. At the indicated days postinfection, five mice per group (except for the day 28 Δm4+m6+m152 group in panel B, which had three mice) were sacrificed and viral titers in the salivary gland were determined by PFU titration on BALB 3T3 cells. Circles indicate individual animals; the lines show the mean per group. Significance was determined by Student's *t* test. Salivary gland titers at 14 days or more postinfection have been compared in five separate experiments with similar results. (C) Mice were infected with 10^6 PFU of tc virus stock, and salivary glands were harvested on day 21 (tc virus). The salivary glands from mice infected with each virus were pooled, yielding salivary gland stock of 1.2×10^5 PFU/ml for wild type and 2.6×10^4 PFU/ml for Δm4+m6+m152 MCMV. Mice ($n = 5$ per group) were infected with 1.5×10^3 PFU of this salivary gland stock, and virus titers in the salivary glands were determined at day 16 postinfection.

restricted epitope from m164 (8). Since T cells specific for each of these epitopes have been shown to recognize wild-type virus-infected cells, particularly when targets are pretreated with IFN-γ (3–5), we first wanted to determine whether the VIPRs had any impact on the presentation of these epitopes. Short-term polyclonal T-cell lines were generated against m164 and pp89 by peptide stimulation of splenocytes from an infected BALB/c mouse and used 11 days later in a standard Cr release assay in the presence or absence of IFN-γ. Figure 1 shows that both pp89-specific and m164-specific cytotoxic T-lymphocyte (CTL) lines readily lysed fibroblasts infected with Δm4+m6+m152, with or without IFN-γ pretreatment. The m164-specific CTL line showed some ability to lyse wild-type virus-infected cells that had been pretreated with IFN-γ. However, in all circumstances, Δm4+m6+m152-infected cells were much more susceptible to CTL than were wild-type-infected cells. Thus, there was a significant quantitative difference in the ability of CD8 T cells to detect the two viruses.

We next infected BALB/c mice with either wild-type or Δm4+m6+m152 MCMV. Mice were sacrificed at various times postinfection, and virus titers in the salivary gland were determined by a standard PFU assay. Figure 2 (A and B) shows the results of two experiments. MCMV was first detected in the salivary gland at 1 week postinfection, at which time there was no significant difference between wild-type and Δm4+m6+m152 virus. At day 14 and all later time points, wild-type virus reached titers that were at least 1 log higher than those of Δm4+m6+m152. These experiments were performed using tissue culture (tc)-prepared virus stocks. To determine whether this difference would persist after tissue adaptation of the viruses, we generated salivary gland stocks from mice infected with tc virus in the experiment shown in Fig. 2C (left side). Naïve mice were then infected with 1.5×10^3 PFU of these stocks, and salivary gland titers were assessed 16 days later (Fig. 2C, right side). Again, wild-type virus titers were more than a log higher than those of Δm4+m6+m152.

To confirm that the defect in Δm4+m6+m152's ability to grow in the salivary gland was due to CD8 T-cell control and not due to an unrecognized mutation elsewhere in the genome, we determined whether CD8 T-cell depletion could eliminate the difference between wild-type and Δm4+m6+m15 viruses. Mice were depleted of CD8 T cells using two monoclonal antibodies directed against the beta chain of CD8. Figure 3 shows that, as previously described, CD8 T-cell depletion did not affect the salivary gland titers of wild-type virus. However, in CD8 T-cell-deficient animals, Δm4+m6+m152 achieved the same titers in the salivary gland as wild-type MCMV did. Thus, the defect in Δm4+m6+m152 growth in the salivary gland is caused by increased susceptibility to CD8 T-cell control.

These results indicate that MCMV in the salivary gland is not intrinsically resistant to the antiviral effects of CD8 T cells. Rather, MCMV's VIPRs apparently act more efficiently in the salivary gland, contributing to the result that CD8 T cells have no impact at this site when they are able to control virus in other organs. We note, however, that even when the VIPRs were removed, CD8 T cells did not rapidly eradicate the virus from the

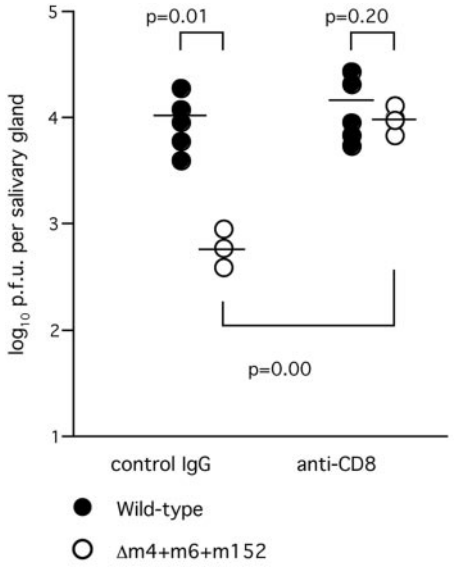

FIG. 3. CD8 T-cell depletion restores growth of Δm4+m6+m152 in the salivary gland to wild-type levels. BALB/c mice were injected intraperitoneally with two monoclonal antibodies against CD8-ß (100 μg 53.5.8, a gift from David Parker, and 100 μg YTS156, a gift from Sophie Sierro) on days −3 and −1 before infection. Control mice were injected with 200 μg rat immunoglobulin G (IgG; Sigma). On day 0, five mice per group were infected with 1×10^5 PFU of wild-type or Δm4+m6+m152 MCMV. Fluorescence-activated cell sorting analysis of two mice showed that CD8 T-cell depletion in the blood was >98% on days 0 and 7 postinfection (data not shown). Mice were sacrificed at day 14 postinfection, and salivary gland virus titers were determined. Circles represent individual animals, and the line shows the mean titer. *P* values were determined by Student's *t* test. Two experiments have been performed with similar results.

salivary gland. Rather, their impact was limited to reducing virus titers. Thus, additional factors, either viral or host encoded, may be involved in the resistance of salivary gland virus to CD8 T-cell control. However, these results suggest that MCMV's VIPRs contribute to the ability of the virus to achieve sufficient titers in the salivary gland for transmission to new hosts after the adaptive immune response has developed.

This work was supported by Public Health Service grant AI-47206 from the National Institute of Allergy and Infectious Diseases.

We are grateful to Ulrich Koszinowski for providing the viruses used and to David Parker and Sophie Sierro for hybridomas.

REFERENCES

1. **Cavanaugh, V. J., Y. Deng, M. P. Birkenbach, J. S. Slater, and A. E. Campbell.** 2003. Vigorous innate and virus-specific cytotoxic T-lymphocyte responses to murine cytomegalovirus in the submaxillary salivary gland. J. Virol. **77:**1703–1717.
2. **Gold, M. C., M. W. Munks, M. Wagner, C. W. McMahon, A. Kelly, D. G. Kavanagh, M. K. Slifka, U. H. Koszinowski, D. H. Raulet, and A. B. Hill.** 2004. Murine cytomegalovirus interference with antigen presentation has little effect on the size or the effector memory phenotype of the CD8 T cell response. J. Immunol. **172:**6944–6953.
3. **Hengel, H., P. Lucin, S. Jonjic, T. Ruppert, and U. H. Koszinowski.** 1994. Restoration of cytomegalovirus antigen presentation by gamma interferon combats viral escape. J. Virol. **68:**289–297.
4. **Hengel, H., U. Reusch, G. Geginat, R. Holtappels, T. Ruppert, E. Hellebrand, and U. H. Koszinowski.** 2000. Macrophages escape inhibition of major histocompatibility complex class I-dependent antigen presentation by cytomegalovirus. J. Virol. **74:**7861–7868.
5. **Holtappels, R., N. K. Grzimek, C. O. Simon, D. Thomas, D. Dreis, and M. J. Reddehase.** 2002. Processing and presentation of murine cytomegalovirus pORF*m164*-derived peptide in fibroblasts in the face of all viral immunosubversive early gene functions. J. Virol. **76:**6044–6053.
6. **Holtappels, R., M. W. Munks, J. Podlech, and M. J. Reddehase.** 2006. CD8 T-cell-based immunotherapy of cytomegalovirus disease in the mouse model of the immunocompromised bone marrow transplant recipient, p. 383–418. *In* M. J. Reddehase (ed.), Cytomegaloviruses: molecular biology and immunology. Caister Academic Press, Wymondham, United Kingdom.
7. **Holtappels, R., J. Podlech, M.-F. Pahl-Seibert, M. Julch, D. Thomas, C. O. Simon, M. Wagner, and M. J. Reddehase.** 2004. Cytomegalovirus misleads its host by priming of CD8 T cells specific for an epitope not presented in infected tissues. J. Exp. Med. **199:**131–136.
8. **Holtappels, R., D. Thomas, J. Podlech, and M. J. Reddehase.** 2002. Two antigenic peptides from genes *m123* and *m164* of murine cytomegalovirus quantitatively dominate CD8 T-cell memory in the *H-2*^{d} haplotype. J. Virol. **76:**151–164.
9. **Jonjic, S., W. Mutter, F. Weiland, M. J. Reddehase, and U. H. Koszinowski.** 1989. Site-restricted persistent cytomegalovirus infection after selective long-term depletion of $CD4^+$ T lymphocytes. J. Exp. Med. **169:**1199–1212.
10. **Koszinowski, U. H., M. Del Val, and M. J. Reddehase.** 1990. Cellular and molecular basis of the protective immune response to cytomegalovirus infection. Curr. Top. Microbiol. Immunol. **154:**189–220.
11. **Krmpotic, A., M. Messerle, I. Crnkovic-Mertens, B. Polic, S. Jonjic, and U. H. Koszinowski.** 1999. The immunoevasive function encoded by the mouse cytomegalovirus gene m152 protects the virus against T cell control in vivo. J. Exp. Med. **190:**1285–1296.
12. **Lucin, P., I. Pavic, B. Polic, S. Jonjic, and U. H. Koszinowski.** 1992. Gamma interferon-dependent clearance of cytomegalovirus infection in salivary glands. J. Virol. **66:**1977–1984.
13. **Pinto, A. K., and A. B. Hill.** 2005. Interference with antigen presentation to CD^+ T cells: lessons from cytomegalovirus. Viral Immunol. **18:**434–444.
14. **Reddehase, M. J., C. O. Simon, J. Podlech, and R. Holtappels.** 2004. Stalemating a clever opportunist: lessons from murine cytomegalovirus. Hum. Immunol. **65:**446–455.
15. **Tortorella, D., B. E. Gewurz, M. H. Furman, D. J. Schust, and H. L. Ploegh.** 2000. Viral subversion of the immune system. Annu. Rev. Immunol. **18:**861–926.
16. **Wagner, M., A. Gutermann, J. Podlech, M. J. Reddehase, and U. H. Koszinowski.** 2002. Major histocompatibility complex class I allele-specific cooperative and competitive interactions between immune evasion proteins of cytomegalovirus. J. Exp. Med. **196:**805–816.
17. **Wagner, M., S. Jonjic, U. H. Koszinowski, and M. Messerle.** 1999. Systematic excision of vector sequences from the BAC-cloned herpesvirus genome during virus reconstitution. J. Virol. **73:**7056–7060.
18. **Yewdell, J. W., and A. B. Hill.** 2002. Viral interference with antigen presentation. Nat. Immunol. **3:**1019–1025.