Comparative Medicine
Copyright 2008
by the American Association for Laboratory Animal Science

Vol 58, No 1
February 2008
Pages 43–46

Development of Breeding Populations of Rhesus Macaques (*Macaca mulatta*) That Are Specific Pathogen-free for Rhesus Cytomegalovirus

Peter A Barry[1–3,*] and Lisa Strelow[1]

Development of breeding colonies of rhesus macaques (*Macaca mulatta*) that are specific pathogen-free (SPF) for rhesus cytomegalovirus (RhCMV) is relatively straightforward and requires few modifications from current SPF programs. Infants separated from the dam at or within a few days of birth and cohoused with similarly treated animals remain RhCMV seronegative indefinitely, provided they are never directly or indirectly exposed to a RhCMV-infected monkey. By systematically cohousing seronegative animals into larger social cohorts, breeding populations of animals SPF for RhCMV can be established. The additional costs involved in expanding the current definition of SPF status to include RhCMV are incremental compared with the money already being spent on existing SPF efforts. Moreover, the large increase in research opportunities available for RhCMV-free animals arguably would far exceed the development costs. Potential new areas of research and further expansion of existing research efforts involving these newly defined SPF animals would have direct implications for improvements in human health.

Abbreviations: HCMV, human cytomegalovirus; NHP, nonhuman primate; RhCMV, rhesus cytomegalovirus; SPF, specific pathogen-free

The impetus for expanding the current SPF definition to include RhCMV is 2-fold. The first is the increasing number of studies involving infection of rhesus macaques with RhCMV as a nonhuman primate (NHP) model of human infection with human cytomegalovirus (HCMV). The second is the recognition that the current SPF protocols result in animals that are also uninfected with RhCMV, such that a relatively minor change in derivation can generate monkeys that meet the current SPF definition and that are uninfected with other endemic viruses, including RhCMV and simian foamy virus.

Natural History of HCMV

HCMV is ubiquitous worldwide, with seroprevalence rates of 50% to 90% in adults.[3] Infection generally does not result in clinical signs of disease in immunocompetent persons, although mild flu-like symptoms and mononucleosis can occur during some primary infections. Like all herpesviruses, HCMV establishes a lifelong persistence characterized by the presence of cells harboring latent viral genomes that periodically and asymptomatically reactivate to produce infectious virus. HCMV is a leading cause of morbidity and mortality in persons who lack a functional immune system, including patients with HIV–AIDS, immunosuppressed transplant recipients, and congenitally infected fetuses. There is no approved vaccine for HCMV, despite repeated calls for such a vaccine and intensive research efforts. Current anti-HCMV drugs are limited in number and generally are characterized by drug-related toxicity, poor bioavailability, and emergence of drug-resistant viral variants. Development of the rhesus macaque model of HCMV has been driven, at least in part, by the desire to investigate viral mechanisms of persistence and pathogenesis in addition to evaluation of protective intervention modalities in a relevant NHP host. Because infection with HCMV is essentially species-specific, utility of NHP as a model for HCMV requires the use of the endemic cytomegalovirus species within the particular NHP host. To date, the vast majority of studies in NHP have been conducted by assessing infection of rhesus macaques with RhCMV.

Received: 18 Sep 2007. Revision requested: 6 Nov 2007. Accepted: 11 Nov 2007.
[1]Center for Comparative Medicine, University of California–Davis, [2]California National Primate Research Center, [3]Department of Pathology & Laboratory Medicine, University of California–Davis, Davis, CA.
[*]Corresponding author. Email: pabarry@ucdavis.edu

Natural History of RhCMV

Infection of rhesus macaques with RhCMV strongly recapitulates the natural history of HCMV.[2] RhCMV is endemic in both captive and wild populations of macaques, with seroprevalence rates approaching 100% by 1 y of age.[8,20] Infection in immunocompetent monkeys is subclinical after either natural exposure to virus-infected animals or experimental inoculation. Although antiviral immune responses effectively limit the disease potential of RhCMV, animals can excrete virus for years after primary infection, again in the absence of disease.[1,7] Virus is detected in saliva and urine frequently, and other potential sources of infectious virus include breast milk and semen. Unlike in humans, vertical transmission of RhCMV has never been documented, although this lack of documentation does not exclude the possibility. Rather, the level of congenital infection, if it does occur, probably was below the level of detection in the extremely few studies in which vertical transmission was addressed.[20] As with HCMV, RhCMV is highly pathogenic in animals coinfected with SIV and in fetuses experimentally inoculated with RhCMV.[2] In these cases, the replication and sequellae of RhCMV are almost identical to those induced by HCMV in comparable clinical settings. Transplant-associated reactivation of cytomegaloviruses

in NHP has occurred only sporadically, mostly involving species other than rhesus macaques. In addition to strong similarities of persistence and pathogenesis, the RhCMV and HCMV genomes exhibit strong DNA and protein sequence identities, befitting the close evolutionary relationship of their respective hosts.[5,14,15] However, each cytomegalovirus species has coevolved with its host, and genetic differences between the cytomegaloviruses isolated from monkeys and those isolated from humans and apes do exist, suggesting that there are distinctions between the virus-host relationships of RhCMV-infected macaques and HCMV-infected humans.

Despite the high seroprevalence of RhCMV in breeding colonies, sufficient numbers of RhCMV-seronegative animals have been obtained for individual research projects. Juvenile macaques (approximately 6 mo) can be screened serologically to identify uninfected animals, estimated to be approximately 50% of the animals surveyed, given the incidences in a previous study.[20] In addition, some nonterminal studies of infant rhesus macaques entail separation of infants from the dams at or within days of birth and physical separation from infected animals. At the completion of such studies, seronegative animals can sometimes be transferred to a study that requires RhCMV-free animals or can be transferred back to the breeding population, thus avoiding purchase costs. This policy may vary among National Primate Research Centers but should be a point of discussion when designing experiments. In recent years, increasing numbers of both investigators and funded projects have used the RhCMV model. Continued moderate growth likely will increase the demand for RhCMV-seronegative monkeys.

One prominent issue with the RhCMV model is that the associated costs for NHP studies far exceed those with small-animal models of HCMV. Accordingly, great care must be taken to maximize the potential for statistically significant results while minimizing costs for the study. The strengths of each animal model are complementary; taken together, they synergize to enable a path of discovery and translational research that ultimately can lead to human clinical trials. Therefore, precedents established by using other models can contribute to the design of experiments in rhesus macaques.

Minimizing costs and ensuring a sufficient supply of animals can be accomplished through several scenarios. One is that, as mentioned earlier, RhCMV-negative animals from nonterminal studies can be transferred to another investigator's project or back to the colony pool of animals, thus reducing or eliminating purchase costs. This scenario might require only additional per diem costs, and use of the NHP resource is maximized. Another scenario uses non-SPF animals to supply the growing demand of studies in which RhCMV serostatus is not a consideration. Some NHP experiments can take advantage of the ability of RhCMV to reinfect previously infected hosts. Moreover, certain vaccine studies can use both naïve and infected animals to assess protective efficacy against horizontally transmitted virus. Finally, expanding the census of RhCMV-seronegative monkeys would ensure a steady supply of animals to meet the projected increased demand while stabilizing animal costs. This expansion is a simple extension of current SPF programs.

Obtaining SPF Animals

Current SPF approach. Physical separation of newborns and dams followed by cohousing with similarly isolated infants forms the basis of current SPF programs. Repeated seroscreening for herpes B virus, simian retrovirus, simian T-lymphotropic virus, and SIV determines which animals remain in the SPF program. At 1 to 2 y of age, animals are housed in larger groups (20 to 30 animals), with 1 to 2 adults for socialization purposes. This procedure has been highly effective at increasing numbers of animals with canonical SPF status. It was recognized at the California National Primate Research Center that juvenile animals remain seronegative for RhCMV as long as there was no direct contact between them and RhCMV-infected animals or their bodily fluids, such as saliva or urine.[2] However, once SPF juveniles are cohoused with an adult SPF animal, the percentage of juveniles that remain seronegative for RhCMV progressively declines, since RhCMV is endemic in the SPF colony. Because macaques continue to excrete RhCMV for years after primary infection, seronegative infants are infected through horizontal transmission of RhCMV from the RhCMV-infected SPF adult. The RhCMV infection rapidly spreads throughout the remaining seronegative juveniles. Transmission can occur within days of cohousing, and every animal seroconverts within months.

Expanded SPF approach. A modified SPF protocol at the California National Primate Research Center has resulted in 3 breeding-age cohorts (approximately 100 rhesus macaques each) of RhCMV-seronegative SPF animals (that is, expanded SPF). In this modified SPF protocol, neonatal monkeys are removed from the dam on the day of birth and reared by hand. Progressively larger cohorts of animals are generated without an adult ever being introduced into the group. Animals are screened repeatedly by ELISA for IgG antibodies to RhCMV antigens[12] by using a single blood draw that is also used for assessment of the multiple viral agents in the expanded SPF definition. As the expanded SPF population increases, adult RhCMV-seronegative animals can be introduced into the larger groups of RhCMV-seronegative juveniles to enhance the socialization process. This procedure probably results in animals that are simultaneously seronegative for all known rhesus macaques herpesviruses, as well as for simian foamy virus, SV40, and many other endemic viruses. Three cohorts of expanded SPF animals have been established in outdoor corrals at the California National Primate Research Center. Successful matings have occurred in 1 of 3 corrals, resulting in the birth of 27 seronegative infants born to seronegative dams; the other 2 corrals are just now going through their first breeding season. This modified process entails a 2-tier SPF program, requiring additional costs for separate housing, serologic screening, and implementation of colony management practices that prevent retrograde transfer of pathogens from nonSPF to conventional SPF to expanded SPF animals. The critical question regarding growth in the number of animals meeting the expanded SPF definition is whether the costs are justified either to meet a demand or to improve animal health and welfare. Answering these questions requires consideration of how these animals would facilitate studies into critical unanswered questions about HCMV and whether RhCMV negatively affects the growth, development, and aging of rhesus macaques.

NHP Modeling of Salient Features of HCMV

A cogent argument can be made that the most pressing issue of HCMV is the lack of an approved vaccine for 2 seronegative populations: women of childbearing age and transplant recipients.[9] To appreciate the long-standing clinical need for a protective vaccine, calls for development of a HCMV vaccine began

soon after HCMV was recognized as an infectious threat to the fetus—37 y ago.[21,22] The Institute of Medicine placed an HCMV vaccine into its highest priority for development because the benefits of a vaccine, primarily preventing the devastating impact of congenital HCMV infection, would far exceed the development costs.[16] Considerable research effort has been expended on vaccine development, and clinical trials are ongoing. However, the natural history of HCMV and the enormous logistic and financial requirements to test protective efficacy have conspired to make development of a vaccine a formidable challenge. The NHP model is well-positioned currently to rigorously test certain aspects of vaccine design and protection.[23] One critical aspect of the NHP model is lacking as yet, namely sufficient numbers of seronegative animals. However, this lack would be obviated by an increased supply of seronegative breeding animals.

The primary goal of a vaccine of which seronegative women of childbearing age would be the target population is prevention of transplacental transmission of HCMV after either primary or nonprimary infection of the woman. At present, vertical transmission cannot be modeled in rhesus macaques, because all breeding-age females are seropositive for RhCMV. For rhesus macaques to be used in trials to assess the protective efficacy of vaccines, 2 advances have to be made. The first is the experimental demonstration that inoculation of seronegative pregnant dams efficiently results in fetal infection. RhCMV rapidly disseminates via the blood to sites throughout the body within 7 to 14 d after inoculation, and there is little doubt that RhCMV would reach the maternal–fetal interface and cross the placenta. Because the fetal central nervous system is acutely sensitive to the devastating effects of intrauterine RhCMV, vaccine modalities that reduce fetal infection and disease likely would be evaluated readily.

The second advance required for their use in vaccine trials is the availability of sufficient seronegative female rhesus macaques of breeding age. Removal of breeding females from such a population would be an expensive venture, the costs of which could be mitigated in part by a large pool to draw from. Although no one can predict how funding agencies might view a fetal transmission model, the 'Holy Grail' for HCMV vaccines is preventing congenital infection. The primary question researchers in academia and industry ask about the rhesus macaque model is whether vertical transmission occurs in monkeys. Assuming that vertical transmission can be demonstrated, the frequency of this question supports the idea that there would be sufficient interest to warrant development of an expanded SPF census. Such a population would have to reach a steady-state that would maintain a threshold size while enabling removal of a certain number of breeding age females each year. Should this aspect of the rhesus macaques model be developed, it would represent a critical NHP adjunct to the only other vertical transmission model for CMV, namely the guinea pig. Moreover, the rhesus macaque model most likely would be essential for clinical trials in humans and should attract both government and corporate funding support.

Another target group for HCMV vaccines consists of HCMV-seronegative transplant recipients receiving tissues or bone marrow from seropositive donors.[9] HCMV is the primary infectious threat to this population. There has been no model development of transplant-associated RhCMV disease in rhesus macaques. There have been anecdotal reports of cytomegaloviral disease in immunosuppressed NHP, mostly involving non-rhesus macaques species.[2] Notably absent are studies assessing primary RhCMV infection under conditions mimicking HCMV infection in patients on antirejection regimens. Intense immunosuppression regimes, particularly those involving antithymocyte globulin, have been reported to be associated with reactivation of cytomegalovirus in seropositive animals.[2] Therefore, whether vaccination of seronegative macaques protects from primary RhCMV infection in antithymocyte-globulin–based immunosuppressed animals likely can be addressed. Development of this aspect of the rhesus macaques model would greatly be enhanced by increased availability of RhCMV-seronegative animals.

Impact of RhCMV on the Health of Rhesus Macaques

The original incentive for derivation of SPF monkeys was to eliminate the zoonotic threat to humans posed by herpes B virus and the disease threat to the animals themselves by simian retrovirus, SIV, and potentially simian T-lymphotropic virus.[6] Implicit in the call to expand the definition of SPF to include RhCMV is the assumption that the health of the newly defined SPF animals will not be compromised and may, in fact, by improved by a reduction in their infectious burden. Infection of rhesus macaques with RhCMV, either after natural exposure to RhCMV-infected animals or by experimental inoculation with high titers of virus, is asymptomatic, consistent with the interpretation that host antiviral immune responses effectively limit the potential for RhCMV to cause disease. In addition, there has never been any association of RhCMV-induced disease in a human exposed to RhCMV. Considering the countless hours of contact of humans with either RhCMV-infected animals or their bodily fluids, the zoonotic disease potential likely can be considered to be exceedingly low. There is no overt evidence at this early stage to suggest that the health and well-being of the 3 expanded SPF cohorts at the California National Primate Research Center are distinguishable from the current SPF or non-SPF populations. Therefore, expansion of the SPF definition probably will not be justified in light of improvements in animal health or reduction in zoonotic potential. However, recent work on HCMV suggests that expansion of the SPF definition may create novel research opportunities with direct translation to human health.

HCMV has been implicated, but not proven, as a contributing factor in development of several chronic conditions in humans, including immune senescence in the elderly humans and chronic inflammation-associated diseases.[10,13] Immune senescence refers to the age-related decline in immune responsiveness to vaccines and infectious agents, particularly in some healthy elderly individuals. Current hypotheses concerning the role of HCMV in development of immune senescence is that the extraordinary devotion of the host's immune repertoire to this single pathogen may preclude an effective immune response to novel antigens, such as influenza or the influenza vaccine, later in life.

Several studies have demonstrated a possible association between the presence of multiple infectious agents, particularly cytomegalovirus, *Chlamydia pneumoniae*, and *Helicobacter pylori*, and the risk of atherosclerosis and cardiovascular disease.[11,24,25] Other studies have not detected a link between infectious burden and cardiovascular events. However, additional studies have observed that in patients with preexisting cardiovascular conditions, prior infection with HCMV is associated with secondary atherosclerosis and restenosis.[4] Similarly, HCMV has been postulated

to be a critical factor in development of atherosclerosis in heart transplant recipients.[19] Tissue culture studies[18] and small animal models of CMV[17] have presented strong evidence for a causal role in vasculopathies. These putative links between HCMV and either immune senescence or cardiovascular disease need further studies to validate or invalidate whether HCMV plays a role. Expansion of the SPF definition to include animals that are seronegative for RhCMV enables a unique opportunity to address these issues on an increasingly large scale.

Development of Novel Model Systems

As RhCMV-seronegative animals increase in number, determining whether the expanded SPF population and non-SPF animals show age-related differences in host immunologic or physiologic parameters likely will be possible. If such distinctions occur and recapitulate those of aging or at-risk humans, then new and unique models of human clinical conditions would be possible in NHP. The National Primate Research Centers are federally funded institutions whose mission is to enable the use of NHP as models of human disease and therapies by providing the NHP resource and expertise. In the current funding climate for NIH-funded grants, it would be prudent for the National Primate Research Centers to develop new NHP models, which could be available to a greater number of investigators. Expansion of the SPF definition to include RhCMV and other viruses, and generation of a stable supply of animals would go a long way to providing a novel resource that can be used by an increased number of academic and industry investigators.

Acknowledgments

This work was supported by funding from the National Institutes of Health (AI063356 and AI49342-06 to PAB) and the California National Primate Research Center (RR000169).

References

1. **Asher DM, Gibbs CJ Jr, Lang DJ, Gadjusek DC, Chanock RM.** 1974. Persistent shedding of cytomegalovirus in the urine of healthy rhesus monkeys. Proc Soc Exp Biol Med **145:**794–801.
2. **Barry PA, Chang W-LW.** 2007. Primate betaherpesviruses. In: Arvin A, Campadelli-Fiume G, Mocarski E, Moore PS, editors. Human herpesviruses: biology, therapy, and immunoprophylaxis. Cambridge (UK): Cambridge University Press.
3. **Britt W.** 2006. Human cytomegalovirus infections and mechanisms of disease. In: Reddehase MJ, editor. Cytomegaloviruses: molecular biology and immunology. Wymondham (UK): Caister Academic Press.
4. **Danesh J, Collins R, Peto R.** 1997. Chronic infections and coronary heart disease: is there a link? Lancet **350:**430–436.
5. **Hansen SG, Strelow LI, Franchi DC, Anders DG, Wong SW.** 2003. Complete sequence and genomic analysis of rhesus cytomegalovirus. J Virol **77:**6620–6636.
6. **Huff JL, Barry PA.** 2003. B-virus (*Cercopithecine herpesvirus 1*) infection in humans and macaques: potential for zoonotic disease. Emerg Infect Dis **9:**246–250.
7. **Huff JL, Eberle R, Capitanio J, Zhou S-S, Barry PA.** 2003. Differential detection of B virus and rhesus cytomegalovirus in rhesus macaques. J Gen Virol **84:**83–92.
8. **Jones-Engel L, Engel GA, Heidrich J, Chalise M, Poudel N, Viscidi R, Barry PA, Allan JS, Grant R, Kyes R.** 2006. Temple monkeys and health implications of commensalism, Kathmandu, Nepal. Emerg Infect Dis **12:**900–906.
9. **Khanna R, Diamond DJ.** 2006. Human cytomegalovirus vaccine: time to look for alternative options. Trends Mol Med **12:**26–33.
10. **Koch S, Larbi A, Ozcelik D, Solana R, Gouttefangeas C, Attig S, Wikby A, Strindhall J, Franceschi C, Pawelec G.** 2007. Cytomegalovirus infection: a driving force in human T cell immunosenescence. Ann N Y Acad Sci **1114:**23–35.
11. **Liu R, Moroi M, Yamamoto M, Kubota T, Ono T, Funatsu A, Komatsu H, Tsuji T, Hara H, Hara H, Nakamura M, Hirai H, Yamaguchi T.** 2006. Presence and severity of *Chlamydia pneumoniae* and *Cytomegalovirus* infection in coronary plaques are associated with acute coronary syndromes. Int Heart J **47:**511–519.
12. **Lockridge KM, Sequar G, Zhou SS, Yue Y, Mandell CP, Barry PA.** 1999. Pathogenesis of experimental rhesus cytomegalovirus infection. J Virol **73:**9576–9583.
13. **Ludewig B, Krebs P, Scandella E.** 2004. Immunopathogenesis of atherosclerosis. J Leuk Biol **76:**300–306.
14. **Oxford KL, Eberhardt MK, Yang K-W, Strelow L, Kelly S, Zhou S-S, Barry PA.** 2008. Protein coding content of the ULb' region of wild-type rhesus cytomegalovirus. J Genl Virol Forthcoming.
15. **Rivailler P, Kaur A, Johnson RP, Wang F.** 2006. Genomic sequence of rhesus cytomegalovirus 180.92: insights into the coding potential of rhesus cytomegalovirus. J Virol **80:**4179–4182.
16. **Stratton KR, Durch JS, Lawrence RS.** 2000. Vaccines for the 21st century: a tool for decisionmaking. Washington (DC): National Academy Press. p 16–172.
17. **Streblow DN, Kreklywich CN, Smith P, Soule JL, Meyer C, Yin M, Beisser P, Vink C, Nelson JA, Orloff SL.** 2005. Rat cytomegalovirus-accelerated transplant vascular sclerosis is reduced with mutation of the chemokine receptor R33. Am J Transplant **5:**436–442.
18. **Streblow DN, Soderberg-Naucler C, Vieira J, Smith P, Wakabayashi E, Ruchti F, Mattison K, Altschuler Y, Nelson JA.** 1999. The human cytomegalovirus chemokine receptor US28 mediates vascular smooth muscle cell migration. Cell **99:**511–520.
19. **Valantine HA.** 2004. The role of viruses in cardiac allograft vasculopathy. Am J Transplant **4:**169–177.
20. **Vogel P, Weigler BJ, Kerr H, Hendrickx A, Barry PA.** 1994. Seroepidemiologic studies of cytomegalovirus infection in a breeding population of rhesus macaques. Lab Anim Sci **44:**25–30.
21. **Weller TH.** 1971. The cytomegaloviruses: ubiquitous agents with protean clinical manifestations (first of two parts). N Engl J Med **285:**203–214.
22. **Weller TH.** 1971. The cytomegaloviruses: ubiquitous agents with protean clinical manifestations (second of two parts). N Engl J Med **285:**267–274.
23. **Yue Y, Kaur A, Eberhardt MK, Kassis N, Zhou SS, Tarantal AF, Barry PA.** 2007. Immunogenicity and protective efficacy of DNA vaccines expressing rhesus cytomegalovirus glycoprotein B, phosphoprotein 65-2, and viral interleukin 10 in rhesus macaques. J Virol **81:**1095–1109.
24. **Zhu J, Nieto FJ, Horne BD, Anderson JL, Muhlestein JB, Epstein SE.** 2001. Prospective study of pathogen burden and risk of myocardial infarction or death. Circulation **103:**45–51.
25. **Zhu J, Quyyumi AA, Norman JE, Csako G, Waclawiw MA, Shearer GM, Epstein SE.** 2000. Effects of total pathogen burden on coronary artery disease risk and C-reactive protein levels. Am J Cardiol **85:**140–146.