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ORIGINAL ARTICLE

A mouse model linking viral hepatitis and salivary gland dysfunction

LM Kasman[1], LL London[1,2], SD London[1,2], MJ Pilgrim[1,3]

[1]*Department of Microbiology and Immunology, Medical University of South Carolina, Charleston, SC, USA;* [2]*Department of Oral Biology and Pathology, School of Dental Medicine, Stony Brook University, Stony Brook, NY, USA;* [3]*Division of Mathematics, Science, and Physical Education, Coastal Georgia, Community College, Brunswick, GA, USA*

OBJECTIVE: Viral hepatitis is known to cause xerostomia in humans, but this has not been reported in an animal model. We report a severe, acute, highly reproducible saliva deficiency occurring in BALB/c mice as a result of experimental viral hepatitis.

MATERIALS AND METHODS: BALB/c mice, splenectomized or carrying genetic mutations to detect immunological contributions to the saliva deficiency syndrome, were infected intraperitoneally with a non-lethal dose of murine cytomegalovirus. Pilocarpine-stimulated saliva volumes were determined between 0 and 15 days after infection. Salivary gland, liver, spleen, and sera were analyzed for the presence of virus, cytokines, inflammatory infiltrates, and tissue damage.

RESULTS: Saliva deficiency was detectable 2 days after cytomegalovirus infection, peaked at 88% below normal by day 7, and resolved partially in all mice by 15 days postinfection as sialoadenitis increased. Neither salivary gland viral titers, sialoadenitis, splenectomy, nor systemic inflammatory markers correlated with hyposalivation severity. Elevated liver enzymes did correlate with hyposalivation, and mice genetically resistant to murine cytomegalovirus-induced hepatitis were significantly protected.

CONCLUSIONS: Murine cytomegalovirus-induced salivary gland dysfunction is biphasic, with an acute hepatitis-associated phase and a later sialoadenitis-associated phase. Acute murine cytomegalovirus infection of BALB/c mice may provide a model for investigation of hepatitis-associated xerostomia.

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Keywords: xerostomia; hyposalivation; hepatitis; cytomegalovirus

Correspondence: LM Kasman PhD, Department of Microbiology and Immunology, Medical University of South Carolina, BSB-201, PO Box 250504, 173 Ashley Ave, Charleston, SC 29403, USA. Tel: +1 843 792 8117, Fax: +1 843 792 2464, E-mail: kasmanl@musc.edu

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Introduction

Salivary deficiency is a common disorder with serious negative consequences for oral health and general physiological well-being (Astor, *et al* 1999, Reznik 2005). Many disease conditions and medications are known to cause hyposalivation, yet a substantial portion of cases have no known etiology (Greenspan, 1996). Saliva deficiency associated with liver damage is such a condition. Hepatitis C infection is often associated with hyposalivation, but the connection between viral hepatitis and saliva deficiency is not understood (Coates *et al*, 2000; Henderson *et al*, 2001). Hepatitis C virus is not found in affected salivary glands, and salivary gland inflammation is only occasionally found in affected people (Verbaan *et al*, 1999; Lins *et al*, 2005). Methamphetamine abuse is also associated with severe xerostomia of unknown etiology, and produces liver damage that mimics viral hepatitis (Dykhuizen *et al*, 1995; Hamamoto and Rhodus, 2009). Therefore, an animal model linking saliva deficiency with hepatitis would be useful to understanding these conditions.

Cytomegalovirus is a common beta-herpesvirus that can cause hepatitis and also displays tropism for the salivary gland (Bolger *et al*, 1999; Campbell, 1999; Varani and Landini, 2002; Krmpotic *et al*, 2003). During murine cytomegalovirus (MCMV) infection, replication of the virus in the salivary gland late in infection stimulates accumulation of extensive lymphocytic infiltrates, primarily in the submandibular gland (Henson, 1972; Cavanaugh *et al*, 2003, Pilgrim *et al*, 2007). This characteristic of MCMV infection has been used in combination with various mouse strains to produce models of salivary gland inflammation and dysfunction (Fleck *et al*, 1998, 2001, Yamano *et al*, 1999; Cavanaugh *et al*, 2003; Ohyama *et al*, 2006). In these models, salivary deficiency is linked with the presence of sialoadenitis, both temporally and mechanistically. It is also well established in BALB/c mice, that the onset of sialoadenitis after MCMV infection parallels the presence of viral replication in the gland, so that infiltrates are not usually detected in the gland prior to day 7 after infection, and peak in severity between

days 14 and 21 (Henson and Strano, 1972; Cavanaugh *et al*, 2003, Pilgrim *et al*, 2007). The effect of MCMV infection on salivary function in mice prior to the onset of sialoadenitis has not been described.

In this report, we demonstrated that an intraperitoneal MCMV infection resulted in a severe saliva deficiency detectable in BALB/c mice within 2 days of infection, before virus or inflammatory infiltrates were present in the gland. This suggested that MCMV-induced salivary gland dysfunction was biphasic: with a rapid first phase, which is independent of salivary gland viremia and sialoadenitis, and a long-term second phase characterized inflammation as previously described by others and associated with sialoadenitis (Fleck *et al*, 1998, 2001; Ohyama *et al*, 2006). The objective of this study was to determine the source of the acute phase pathology in the BALB/c MCMV model.

Materials and methods

Reagents

Pilocarpine nitrate was purchased from Sigma. X-Gal (5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside) was from Gold Biotechnology (St. Louis, MO, USA). Gibco minimal essential medium and other tissue culture reagents were purchased from Invitrogen, Inc. (Carlsbad, CA, USA) or Cambrex (Rutherford, NJ, USA).

Animals

Five-week-old, female BALB/cByJ mice (BALB/c-WT) were purchased from Jackson Laboratories (Bar Harbor, ME, USA). B6.BALB-Cmv1^{r} mice (Scalzo *et al*, 1999) were produced in the MUSC animal facility by mating a C57BL/6 female (*Cmv1^{r}* homozygote) with a BALB/cByJ male (*Cmv1^{r}* negative), and backcrossing female offspring carrying the *Cmv1^{r}* genetic marker, D6Ott151, with BALB/cByJ males for five generations. Sixth generation offspring heterozygous for the *Cmv1^{r}* gene were used in these experiments. Genotyping for the *Cmv1^{r}* gene was performed by PCR amplification from tail genomic DNA using primers D6Ott151-F: 5′-GTG CTA CCA CTG AAA ACC ATT G-3′ and D6Ott151-R: 5′-CTG TCT CTT GAG TCA CCT GCA C-3′ according to a protocol kindly provided by Seung-Hwan Lee and S. Vidal (University of Ottawa) (Lee *et al*, 2003). Provisionally congenic BALB/cByJ-*c-Rel* knock-out mice (BALB/c-Rel$^{-/-}$ in this report) were also bred in the MUSC animal facility by repeated backcrossing of C57BL/6-*c-rel* knock-out female mice generously provided by Kendall A. Smith and Hsiou-Chi Liou (Weill Medical College of Cornell University, New York, NY, USA) (Liou *et al*, 1999) with BALB/cByJ males for five generations and then brother–sister matings of fifth generation offspring to produce homozygous *c-Rel* knock-outs on a BALB/cByJ background. Second generation homozygous *c-Rel* knock-outs were used in these experiments. Genotyping for the *c-Rel* gene utilized primers Rel-forward [5′-ATA GAA GTT CGT TTT GTG TTG AA-3′] and Rel-reverse [5′-TCA CTA ACT TCC TGG TCA GAA GG-3′] for the wild-type allele and Rel-forward and Rel-neomycin for the knock-out allele [5′-CTC TCG TGG GAT CAT TGT TTT TC-3′], with the protocol provided by Hsiou-Chi Liou (Cornell University). Splenectomized BALB/c-WT mice were purchased from Jackson Laboratories.

All experiments in this study conform with 'The guide for the Care and Use of Laboratory Animals' published by the US National Institutes of Health, Publication No. 85-23, revised 1996) and were approved by the MUSC institutional committee for the care and use of laboratory animals. Food and water were provided *ad libitum*. All mice were housed in HEPA-filtered cages in the approved animal facility and monitored daily.

Saliva collection

Saliva output at each time point was determined by collection of pilocarpine-stimulated saliva from conscious mice, according to a protocol modified from Lin *et al* (2001). Mice were injected intraperitoneally with 0.1 mg pilocarpine nitrate in 50 μl sterile saline. Exactly 2 min after injection, saliva was collected from the mouth and cheek pouches for 30 s with a 200 μl pipetter while holding the mouse vertically, discharging collected saliva into a 1.5 ml eppendorf tube as needed. After 30 s of collection, the mouse was put down for 30 s. This sequence was immediately repeated six times for a total of seven collection periods of 30 s each, and ending 8.5 min after the pilocarpine injection. Saliva samples were kept on ice until centrifugation at 16 000 ***g*** for 5 min, then transferred to new tubes, 20 μl at a time to determine volumes, and frozen at −80°C.

Virus and infections

Murine cytomegalovirus strain RM461 was generously provided by Edward Mocarski, (Emory University) and was used in all experiments. This virus expresses β-galactosidase late in infection, simplifying plaque assays (Stoddart *et al*, 1994). Virulent virus was prepared by salivary gland passage as described previously (Hamamdzic *et al*, 2001). Mice were infected at 5–6 weeks of age by intraperitoneal injection of 10^5 PFU of salivary gland-passaged virus in a volume of 0.1 ml using minimal essential medium as a diluent. Sham-infected mice were injected with an equivalent volume of uninfected salivary gland homogenate in the same diluent. After final saliva collection, the animals were killed by CO_2 overdose. Spleens, livers, salivary glands and blood (serum) via cardiac puncture (from heart blood) were obtained immediately postmortem and stored frozen at −70°C until further analysis. Some tissues were divided and one half fixed for histological analysis.

Histology

Tissues were fixed in 10% neutral buffered formalin v/v (Richard Allen Scientific, Kalamazoo, MI, USA) for 24 h at 22°C before being embedded in paraffin. Hematoxylin and eosin staining were performed on 4-μm sections. Images at 40× magnification on an Olympus B×40 microscope (Olympus, Melville, NY, USA) were captured with a Polaroid digital microscope

camera and edited using Graphic Converter (Lemke Software GmBH, Peine, Germany). Salivary gland pathology was graded by a blinded observer according to the following scale: (−) no difference from uninfected control, (+) normal tissue architecture, occasional inflammatory cells in field, (++) tissue architecture disrupted, separate inflammatory foci in field, (+++) tissue architecture disrupted, and inflammatory foci consolidation.

MCMV titering

Tissues were dounce homogenized using 25 strokes in 0.5 ml cold Modified Eagle Medium containing 0.1% porcine gelatin and 10% DMSO. After centrifugation for 2 min at 16 000 ***g***, supernatants were aliquoted and frozen at −70°C for titer determinations. Virus titers were determined on murine 3T3 fibroblasts as described previously (Kasman, 2005), except that 24-well plates were used and cells were seeded at a rate of 6×10^4 per well.

Serum analyses

Raybiotech Mouse Cytokine Antibody Array I was used to analyze sera from BALB/c-Rel$^{-/-}$, B6.BALB-Cmv1^{r} and BALB-WT mice infected for 7 days and BALB-WT mice sham infected for 7 days. Sera from four mice were pooled in equal amounts for each of the four samples tested. Analysis was carried out according to manufacturer's instructions, with final detection by exposure to X-ray film. Serum liver enzymes, albumin, and blood urea nitrogen (BUN) levels were determined in individual mouse sera by IDEXX Preclinical Research Services (W. Sacramento, CA, USA).

Immunoblotting

Salivary gland homogenates were normalized for total protein, size-separated on 4–12% NuPAGE gels (Invitrogen) and transferred to nitrocellulose according to manufacturer's instructions. Antibody to aquaporin 5 (LS-C3809) was purchased from Lifespan Biosciences (Seattle, WA) and used at 1:800 dilution. Polyclonal rabbit anti-actin antibody was purchased from Sigma and used at 1:1000. Both were detected with the HRP-conjugated anti-rabbit secondary antibody (1:3000) and ECL substrate kit from Pierce (Woburn, MA, USA). Signal density for each band was determined with a BioRad (Hercules, CA, USA) Gel Doc 2000 system.

Statistical analysis

P-values were determined by ANOVA followed by Tukey's Multiple Comparison Post-test if ANOVA gave a $P < 0.05$, and performed using GraphPad Prism version 3.0cx for Macintosh, GraphPad Software, San Diego, CA, USA, http://www.graphpad.com.

Results

Intraperitoneal injection of MCMV results in transient saliva deficiency in BALB/c mice

To observe the effect of acute MCMV infection on saliva production, groups of 5-week-old, female BALB/cByJ (BALB/c-WT) mice were infected with 10^5 PFU of MCMV, or sham infected, by intraperitoneal injection. Prior to infection (day 0) and on days 2, 5, 7, 9, 12, and 15 postinfection, mice were weighed and saliva was collected for a standardized period following pilocarpine stimulation. The volume of saliva per gram body weight was determined for each collection to control for weight loss associated with viral infection. Mean saliva volumes per treatment group over time (Figure 1a) indicated that saliva deficiency was evident by 2 days postinfection (dpi), was most severe between 6 and 8 dpi, and was resolving between days 9 and 15 dpi. A statistically significant decrease in saliva volume was observed on days 2, 5, 7, and 9 post-MCMV inoculation *vs* uninfected control mice, with a maximum deficiency of 85% (Figure 1a, Table 1). Repeated saliva sampling

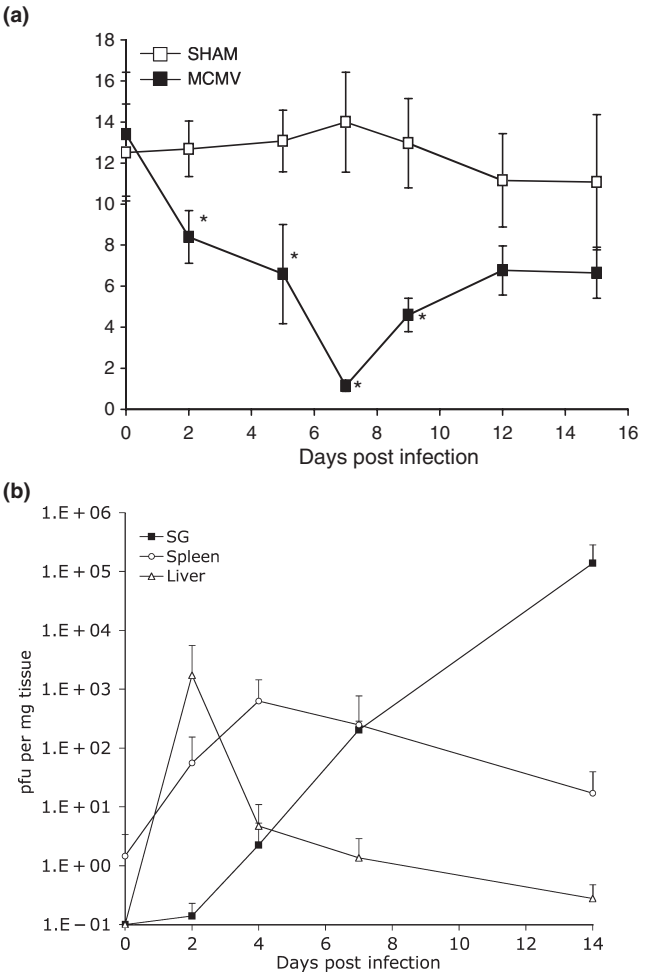

Figure 1 Intraperitoneal injection of MCMV results in transient saliva deficiency in BALB/c mice that peaks between days 6 and 8 postinfection. (**a**) Mean (±s.d.) pilocarpine stimulated saliva volumes per gram body weight collected from 5-week-old female BALB/c-ByJ mice injected intraperitoneally with 10^5 PFU MCMV (filled squares) or an equivalent sham infection (open squares). The figure is representative of two similar experiments, $n = 4$–6 mice per data point. *$P < 0.01$. (**b**) Mean (±s.d.) MCMV titers in salivary glands, livers, and spleens of mice with MCMV-induced saliva deficiency 0–15 days postinfection. Each point represents an average of the viral titer per gram of homogenized tissue of six or more mice

Table 1 Summary of viral titers and tissue pathology for salivary gland and spleen in three mouse strains. Statistical analysis for significant differences between strains was performed by one-way ANOVA

Mouse strain	*Phenotype*	*Saliva deficiency (relative to sham infected), %*	*7 dpi salivary gland titers*[a]	*7 dpi spleen titers*[b]	*7 dpi salivary gland pathology*	*15 dpi salivary gland pathology*	*7 dpi spleen pathology*
BALB/c-wild type	MCMV susceptible	92	8.3E + 03	1.6E + 03	+ +	+ + +	Hemorrhagic, necrotic
BALB/c-c-Rel$^{-/-}$	Inflammation deficient	88	7.4E + 03	6.8E + 04	+ +	+ + +	Lacks follicles, necrotic
B6.BALB-Cmv1^{r}	MCMV resistant	39	5.0E + 03	1.2E + 02	+ +	+ + +	Active germinal centers

dpi, days postinfection.
[a]Plaque forming units per gland, no significant difference between strains.
[b]Plaque forming units per spleen ($P = 0.0001$).

in the same mice did not in itself induce saliva deficiency as saliva production in sham-infected mice increased or remained stable for the seven collections over the 15-day time course.

Dehydration is a well-known cause of transient saliva deficiency, which may occur during acute infections (Ship and Fischer, 1997). To ensure that the observed saliva deficiency was not a result of dehydration, groups of mice were treated with supplementary fluids by injection. Volumes of warm, isotonic saline equivalent to 85% of the average BALB/c mouse daily oral water intake (Bachmanov *et al*, 2002), and representing 8–12% of body weight in these animals failed to reduce the MCMV-induced saliva deficiency, indicating dehydration was not a significant contributor to the syndrome (Figures S1 and S2, Appendix).

Salivary deficiency is not correlated with salivary gland viremia

Cytomegalovirus-associated hyposalivation might be expected as a natural consequence of infection given the virus' tropism for the salivary gland. The glandular epithelial acinar cells of the salivary gland are the primary site of CMV replication and chronic infection, and they are the cells most responsible for the volume of saliva produced (Lucin *et al*, 1992). However, MCMV-induced hyposalivation was not correlated with salivary gland viremia. MCMV titers in salivary gland tissue were extremely low on day 2 at the onset of hyposalivation, while they increased almost 1000-fold between days 7 and 14 when the saliva deficiency was resolving (Figure 1b, SG). In contrast, viral titers in the spleen were the highest when the hyposalivation was most severe (days 2–6), and were decreasing as the salivary deficiency resolved (Figure 1b). Liver titers were also elevated early in the time course, peaking on day 2, prior to peak hyposalivation (Figure 1b).

Salivary deficiency is not correlated with systemic or salivary gland inflammation

While chronic sialoadenitis and salivary gland fibrosis are well documented in susceptible mice weeks to months after initiation of MCMV infection, four lines of investigation indicated that an inflammatory response was not responsible for the observed acute MCMV-associated saliva deficiency peaking 7 days after infection. First, in the BALB/cByJ-wild-type mice, lymphocytic infiltrates indicative of salioadenitis did not correlate in time with saliva deficiency. Sialoadenitis was worse on day 15 during the resolution phase of the saliva deficiency, than on day 7 when saliva production was at its lowest (Figure 2).

Second, mice deficient in the TH1 inflammatory response (*c-Rel* knock-out BALB/cJ mice) were not protected from MCMV-associated saliva deficiency (Table 1, Figure 3a). Transgenic *c-Rel* knock-out mice lack the p65 subunit of NF-KB expressed primarily in hematopoietic cells and are profoundly deficient in TH1 responses (Hilliard *et al*, 2002; Pai and Ho, 2002), rendering them resistant to several experimental inflammatory diseases including experimental autoimmune encephalitis (Hilliard *et al*, 2002), allergic asthma, and experimental rheumatoid arthritis (Donovan *et al*,

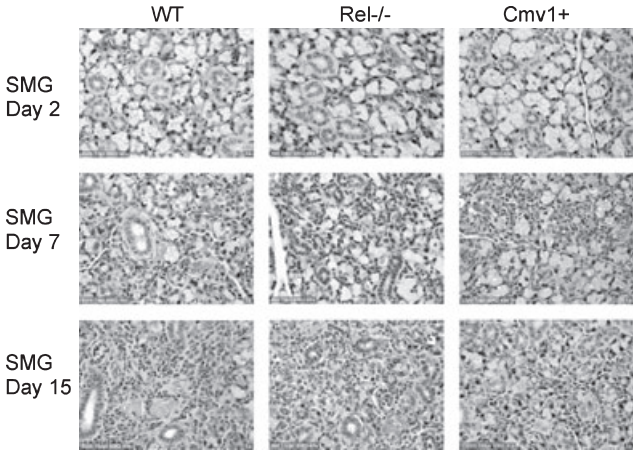

Figure 2 Histology of submandibular salivary glands after MCMV infection in wild-type and mutant BALB/c mice. Formalin-fixed submandibular salivary glands (SMG) on days 2, 7, and 15 postinfection are shown from BALB-WT, BALB/c-Rel$^{-/-}$, and B6.BALB-Cmv1^{r} mice as indicated. Tissues are hematoxylin and eosin stained and photographed at 40× magnification

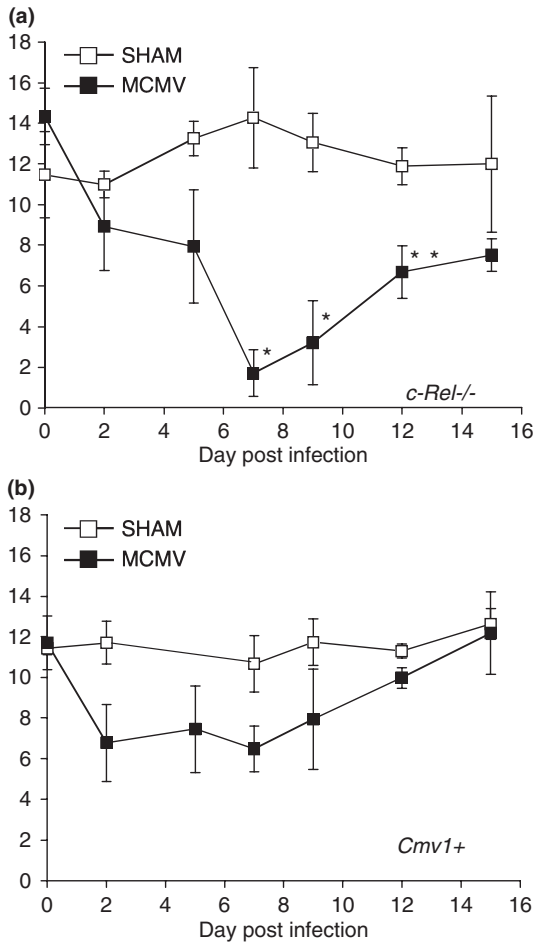

Figure 3 Saliva output of BALB/c-Rel$^{-/-}$, and B6.BALB-Cmv1r^{r} mice 0–15 days after MCMV infection. Groups of four to six 5-week-old female mice of each strain were injected intraperitoneally with 10^5 PFU MCMV or a sham inoculum. Body weights and pilocarpine-stimulated saliva volumes were determined on days 0, 2, 5, 7, 9, 12, and 15 after infection. Mean (±s.d.) volumes of saliva per gram body weight in infected (filled symbols) and sham-infected (open symbols) mice are shown for (**a**) BALB/c-Rel$^{-/-}$ mice (**b**) B6.BALB-Cmv1r^{r} mice. *$P < 0.01$, **$P < 0.05$. The figure is representative of two similar experiments

1999). However, MCMV-induced saliva deficiency was not reduced in the *c-Rel* knock-out animals compared with wild-type animals. Pilocarpine-stimulated saliva production was significantly lower than that of sham-inoculated *c-Rel* knock-out animals on days 7, 9, and 12, and was nearly identical to that observed in infected BALB-WT mice over the 15-day time course (Figure 3a, Table 1). On day 7 postinfection, the period of greatest deficiency in both strains, saliva production was reduced 92% in BALB-WT and 88% in BALB/c-Rel$^{-/-}$ mice, to < 2 μl g^{-1} of body weight in both strains. In addition, as demonstrated with wild-type animals, the time course and severity of MCMV-induced sialoadenitis (lymphocytic infiltrates) did not correlate in time with saliva deficiency and were indistinguishable from that of wild-type mice (Figure 2, Table 1).

Third, MCMV-resistant mice (*Cmv1^{r}* mice) were significantly protected from hyposalivation but not from salivary gland inflammation (Figure 2, Table 1). *Cmv1r* is an extensively characterized murine gene, which confers resistance to MCMV-induced mortality and morbidity, primarily by reducing viral replication in the spleen and liver (Scalzo *et al*, 1992; Lee *et al*, 2002, 2003). *Cmv1^{r}* encodes the NK cell activating receptor Ly49H, and is normally absent in BALB/c mice (Lee *et al*, 2002). Provisionally, congenic B6.BALB-Cmv1^{r} mice, heterozygous for *Cmv1^{r}*, were produced by backcrossing and were used in these experiments. MCMV-induced saliva deficiency in B6.BALB-Cmv1^{r} mice was significantly less severe than that observed in BALB/c-WT mice ($P < 0.01$ at 7 dpi, $P < 0.05$ at 12 and 15 dpi) indicating that the Cmv1^{r} phenotype protected BALB/c mice from MCMV-induced saliva deficiency (Figure 3b). However, as expected, if inflammation has no role in the acute MCMV-induced hyposalivation syndrome, the time course and severity of MCMV-induced sialoadenitis (lymphocytic infiltrates) in B6.BALB-*Cmv1^{r}* mice did not correlate in time with saliva deficiency and were indistinguishable from that of wild-type mice (Figure 2, Table 1).

Fourth, given that gross salivary gland pathology and viral titers could not be linked to severity of saliva deficiency, systemic factors were analyzed. Sera from groups of four mice of each strain at 7 dpi were pooled and screened for the presence of 22 murine inflammatory cytokines on RayBiotech Mouse Inflammatory Antibody Arrays^{TM}. Sera pooled from four sham-infected BALB/c-WT were screened in parallel as a control. The results did not show any changes consistent with a cause of saliva deficiency (Figure S3, Appendix). All sera pools contained detectable levels of IL-12p40p70 and soluble TNF receptor. However, the sera pool from the BALB/c-Rel$^{-/-}$ mice, which had saliva deficiency symptoms similar to those of BALB/c-WT, was the only one to contain detectable amounts of MCP-1, MCP-5, and RANTES. Other factors screened by the array but not detected in this experiment were GCSF, GM-CSF, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12p70, IL-13, IL-17, IFN-gamma, SCF, TNF-alpha, TPO, and VEGF. Saliva and salivary gland homogenates could not be analyzed by this method because of high levels of interfering proteins, including immunoglobulin binding proteins (Wozniak *et al*, 2002).

Aquaporin 5 levels in salivary gland tissue of MCMV-infected mice

Aquaporin 5 is the main water channel expressed in the salivary gland and is crucial for normal salivation (Krane *et al*, 2001). To determine whether aquaporin 5 levels in salivary gland were altered by MCMV infection, whole salivary gland homogenates collected from BALB-WT mice infected 0, 2, 4, 7, 14 and 21 days were subjected to electrophoresis and immunoblotting with an anti-aquaporin 5 antibody. Peyers patch homogenate was run as a negative control for aquaporin 5. Actin levels were probed as a loading control. The ratio of aquaporin 5 to actin signal was determined by densitometric analysis for all time points, and compared over time by setting the ratio on day 0 to 1:1. On the basis of this analysis, aquaporin 5

levels were unchanged on days 0 through 4, then dropped by 30% on day 7, and by more than 75% on days 14 and 21 (Figure 4). There was no indication of a drop in aquaporin 5 levels at the onset of saliva deficiency symptoms on day 2, or at any time before infiltrates were detectable in the gland. Therefore, while relative aquaporin 5 levels did decrease after MCMV infection, the timing was not correlated with saliva deficiency symptoms.

Asplenic mice are not protected from MCMV-induced saliva deficiency

The data presented thus far established that the acute MCMV-associated saliva deficiency syndrome was not correlated with salivary gland viremia or pathology. However, viremia and pathology of the spleen did appear to correlate with saliva deficiency in time and in severity (Figure 1b, Table 1). Average spleen titers at 7 dpi (the period of peak deficiency) were approximately 13 times and 300 times higher in the severely affected BALB/c-WT and BALB/c-Rel$^{-/-}$ mice respectively, than in the protected B6.BALB-Cmv1^{r} mice (Table 1). In addition, spleens of B6.BALB-Cmv1^{r} mice 7 dpi appeared normal with active germinal centers while spleens of severely saliva-deficient BALB/c-WT and BALB/c-Rel$^{-/-}$ mice were markedly abnormal, with necrotic lesions (Table 1). Therefore, to determine the contribution of spleen pathology to the MCMV-induced hyposalivation syndrome, asplenic mice and their intact normal littermates were purchased and evaluated for susceptibility to MCMV-induced saliva deficiency. Interestingly, asplenic mice were not protected from the acute hyposalivation syndrome (Figure 5). Saliva output in infected asplenic mice was indistinguishable from that of infected intact mice. Both infected asplenic and intact mice produced significantly less saliva than sham-infected asplenic mice at days 5, 7, 9, and 15 postinfection ($P < 0.05$), but there was no significant difference in salivary gland output between infected intact and asplenic mice. Figure 5 is the average of two similar experiments ($n = 7$ per treatment group). These results indicate that the acute MCMV-induced saliva deficiency syndrome occurred independently of any contribution of the spleen.

Liver pathology severity, as measured by serum liver function tests, correlates with severity of saliva deficiency

Intraperitoneal MCMV infection produces acute viral hepatitis in BALB/c-WT mice, with peak viral titers in the liver on day 2 postinfection (Figure 1b) coincident with the onset of hyposalivation (Figure 1a). Therefore, to investigate the possible role of liver injury in the MCMV-induced saliva deficiency, complete blood chemistries were performed on sera from susceptible BALB/c-WT and asplenic as well as resistant B6.BALB-Cmv1^{r} mice. Sera from uninfected mice were compared with those from mice 5 dpi, when MCMV hepatitis is most severe (Pilgrim *et al*, 2007). Comparisons of key liver function indicators are shown as fold change over uninfected control sera in Figure 6.

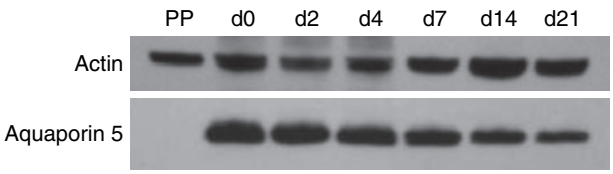

Figure 4 Immunoblot of aquaporin 5 in whole salivary gland homogenates. Salivary gland homogenates from 5-week-old BALB/cByJ female mice infected with 10^5 PFU MCMV for 0, 2, 4, 7, 14, or 21 days as indicated, and Peyer's patch homogenate (PP) as a negative control, were normalized for total protein before loading. The immunoblot was probed sequentially for aquaporin 5 and actin, and the bands compared by densitometry. The result is representative of three similar experiments

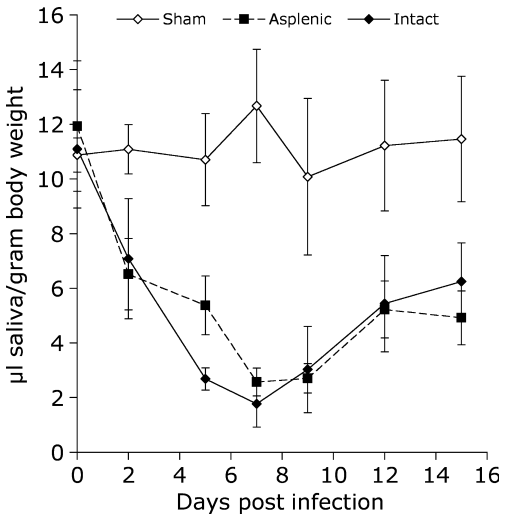

Figure 5 Saliva output of splenectomized BALB/c-WT mice 0–15 days after MCMV infection. Mean (±s.d.) volumes of pilocarpine-stimulated saliva per gram body weight produced by normal intact (dashed line) or splenectomized (solid line) mice that have been sham (open) or MCMV (solid) infected. Differences between sham and infected were significant at days 5, 7, 9, and 15 ($P < 0.05$). There were no significant differences between intact and splenectomized groups. Each data point represents at least seven mice

A number of liver enzymes levels were not dramatically different in the mice susceptible to MCMV-saliva deficiency compared with the resistant B6.BALB-Cmv1^{r} mice. Alkaline phosphatase (ALK), an indicator of bile congestion or liver infiltration was unchanged. Albumin, a measure of serum osmolarity, was not affected by saliva dysfunction status and was only slightly less than sham-infected controls, confirming that dehydration is not a significant factor in the hyposalivation syndrome. Similarly, creatine kinase (CK) and BUN, indicators of muscle and/or kidney damage, were only mildly affected. CK was slightly elevated in asplenic and intact wild-type BALB/c compared with controls, and slightly decreased in B6.BALB-Cmv1^{r}.

In contrast, two key liver function enzymes were dramatically increased in hyposalivating mice. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were elevated more than nine- and fivefold, respectively, in mice displaying saliva deficiency (BALB-asplenic and WT) compared with controls, while being elevated two-fold or less in the B6.BALB-Cmv1^{r} mice that do not experience significant MCMV-induced saliva deficiency. ALT and AST indicate hepatocyte damage resulting in leakage of cell contents.

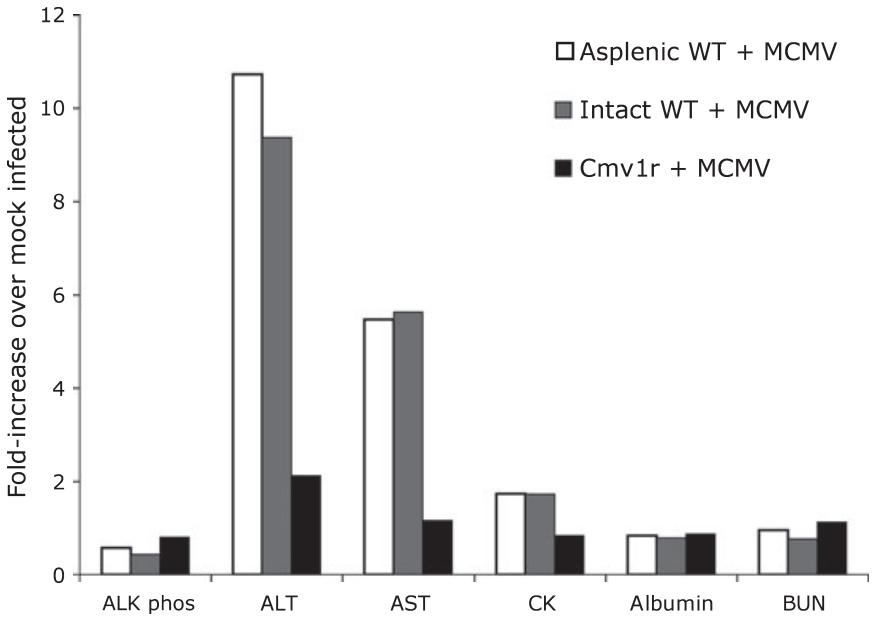

Figure 6 Blood chemistry analysis of sera from MCMV-infected mice 5 days postinfection. Sera from asplenic BALB/c-WT, normal (intact) BALB/c-WT, and B6.BALB-Cmv1^{r} mice killed on day 5 post-MCMV infection, as well as from uninfected BALB-WT were analyzed for levels of alkaline phosphatase (ALK Phos), alanine amino transferase (ALT), aspartate amino transferase (AST), creatinine kinase (CK), albumin, and blood urea nitrogen (BUN) ($n = 2$). Each bar represents an average fold increase in values from sera of infected mice relative to the average from sham-infected controls (average value for infected mice/average value for sham-infected mice)

Discussion

Saliva deficiency is a common disorder, which can be caused by many disease conditions and numerous medications (Greenspan, 1996). The mechanisms associated with saliva deficiency, have fallen into three main categories: (i) physical damage to the salivary gland from infection, trauma, radiation, or autoimmune destruction; (ii) inadequate fluid transport to the salivary glands because of low blood pressure or dehydration, as with anti-hypertensive drugs or prolonged thirst (Ship and Fischer, 1997); or (iii) cholinergic dysfunction of the salivary gland caused by neural damage, anti-cholinergic drugs, or possibly anti-muscarinic receptor autoantibodies. We have described a small animal model of salivary gland deficiency, which occurs after intraperitoneal infection with MCMV and does not appear to fit into any of these categories. Saliva deficiency occurred days prior to the appearance of glandular immunopathology, and was not relieved by rehydration or genetic inactivation of inflammatory pathways. Salivary gland viral titers were not yet detectable at the onset of saliva deficiency, and were the highest when saliva production was improving. Sialoadenitis was also more severe during the resolution phase of the hyposalivation symptoms and was similar in the three mouse strains tested (BALB/c-WT, BALB/c-Rel$^{-/-}$, and MCMV-resistant strain, B6.BALB-Cmv1^{r}) despite large differences in their saliva production. These findings in combination with equal susceptibility in TH1-deficient BALB/c-Rel$^{-/-}$ mice are evidence that the early phase of MCMV-induced salivary gland dysfunction occurs independently from inflammation or viral replication in the salivary gland. Recently, ischemic injury to the salivary gland via ligation was also shown to induce transient saliva deficiency in the absence of inflammation (Correia *et al*, 2008).

The factors most associated with severity of MCMV-associated saliva deficiency in this model were spleen and liver viral titers and inflammation, which were the highest between 2 and 7 days after infection when saliva deficiency was the most severe, and had largely resolved by 15 days when saliva deficiency was subsiding. This suggested that damage to these organs was acting on the salivary gland, potentially via a soluble factor. Several findings identified liver damage as the probable source of this potential factor. Critically, total splenectomy prior to infection had no effect on the acute phase saliva deficiency syndrome. Mice without spleens displayed hyposalivation with an identical time course and severity to intact mice. Second, enzymes associated with liver damage were highly elevated in the sera of affected BALB/c-WT and Rel$^{-/-}$ mouse strains but not in the MCMV-resistant strain, B6.BALB-Cmv1^{r}, which displayed significantly less saliva deficiency. Specifically, ALT levels were elevated in the absence of ALK or CK levels, indicating hepatocyte damage. Third, the B6.BALB-Cmv1^{r} mouse strain is genetically protected from viral replication in the liver and spleen, but not the salivary gland, (Scalzo *et al*, 1990; Lee *et al*, 2001). Finally, an additional indication of a liver toxicity association with saliva deficiency was the finding of elevated MCP-1 and RANTES on the serum cytokine array, cytokines often associated with liver damage (Zamara *et al*, 2007; Karlmark *et al*, 2008).

What soluble factors are candidates for inhibiting stimulated saliva production? It has been shown previously that certain inflammatory cytokines, including TNF and IFN-gamma, can induce acinar cell death *in vitro* (Wu *et al*, 1996; Azuma *et al*, 1997). Sjogren's syndrome of autoimmune destruction of the salivary gland is accompanied by systemic autoantibodies and cytokines (Azuma *et al*, 1997). Therefore, it may be possible for soluble factors to induce glandular damage prior to accumulation of a cellular infiltrate in the

salivary gland. However, several facts rule out autoantibodies or inflammatory cytokines in this case. First, BALB/c-Rel$^{-/-}$ mice have both impaired cytokine and antibody production, yet suffered saliva deficiency equal to that of BALB/c-WT. Second, the rapid onset of hyposalivation was incompatible with specific antibody production. Third, histological sections also showed no changes in ductal architecture in affected mice that would indicate acinar cell death. Fourth, expression of aquaporin 5, the main water transporter expressed in the salivary gland, although known to be down-regulated during adenovirus infection and by interferon-alpha in some models (Smith *et al*, 1999; Towne *et al*, 2000), was not decreased in salivary glands of mice in the acute phase of hyposalivation. Rather its levels decreased slightly relative to actin as saliva production recovered, probably because of infiltration of lymphocytes, which lack the protein.

An alternative possibility is that virus-induced liver damage prevents production of a serum factor or factors that regulate tissue water balance or glandular function, inhibiting salivation. The liver is the primary site of synthesis of multiple blood coagulation factors and components of the innate immune system. Association of non-viral causes of liver damage with saliva deficiency suggests this may be the case. For example, biliary cirrhosis, a chronic autoimmune disease resulting in hepatocyte destruction, often first presents as a sicca syndrome involving the eyes and oral cavity (Mang *et al*, 1997). Habitual users of methamphetamine suffer liver damage 'indistinguishable from viral hepatitis' and also frequently suffer from severe xerostomia (Dykhuizen *et al*, 1995; Fidler *et al*, 1996; Andreu *et al*, 1998). Taken together with the strong association between hepatitis C infection and xerostomia, these syndromes suggest that hepatocyte injury may be a general cause of salivary gland dysfunction.

In conclusion, we have shown that MCMV-induced salivary gland dysfunction has two phases. The previously characterized phase of chronic inflammatory destruction of the salivary gland is preceded by a transient, severe, non-inflammatory saliva deficiency syndrome associated with viral hepatitis. This appears to be the first description of an animal model linking viral hepatitis with salivary deficiency. Further investigation of this model may elucidate the mechanism responsible for liver-associated sicca syndromes in humans.

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Author contributions

Dr Kasman designed the study, performed all experiments except Figure 1b, analyzed the data and wrote the paper. Dr L London advised the first author on data analysis and presentation, and critically edited the paper. Drs Pilgram and S London made the original observation of acute hyposalivation in MCMV infected mice and contributed data in Figure 1b.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Mean (±s.d.) pilocarpine stimulated saliva volumes per gram body weight collected on days 0, 4, and 7 after MCMV infection from 5-week-old female BALB/c-ByJ mice that were also IP injected daily with 0.1 ml saline (light gray bars, control) or 2.0 ml saline (dark gray bars, therapeutic rehydration). No statistically significant differences were found.

Figure S2. Mean (±s.d.) pilocarpine stimulated saliva volumes per gram body weight collected on day 7 after MCMV infection, before (0 h) or 2 h after rehydration by IP injection with 0.1 ml saline (light gray bars, control) or 2.0 ml saline (dark gray bars).

Figure S3. Serum cytokine profiles in mice with MCMV-induced xerostomia.

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