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Severe Focal Sialadenitis and Dacryoadenitis in NZM2328 Mice Induced by MCMV: A Novel Model for Human Sjögren’s Syndrome

Yukiko Ohyama, Virginia A. Carroll, Umesh Deshmukh, Felicia Gaskin, Michael G. Brown, and Shu Man Fu

The genetic and environmental factors that control the development of Sjögren’s syndrome, an autoimmune disease mainly involving the salivary and lacrimal glands, are poorly understood. Viruses which infect the glands may act as a trigger for disease. The ability of sialotropic murine CMV (MCMV) to induce acute and chronic glandular disease was characterized in an autoimmune-prone mouse strain, NZM2328. MCMV levels were detectable in the salivary and lacrimal glands 14–28 days after i.p. infection and correlated with acute inflammation in the submandibular gland. After latency, virus was undetectable in the glands by PCR. At this stage, NZM2328 female mice developed severe chronic periductal inflammation in both submandibular and lacrimal glands in contrast to the much milder infiltrates found in female B6-lpr and male NZM2328. The focal infiltrates consisted of CD4⁺ and B220⁺ cells as opposed to diffuse CD4⁺, CD8⁺, and B220⁺ cells during acute infection. Salivary gland functional studies revealed a gender-specific progressive loss of secretory function between days 90 and 125 postinfection. Latent MCMV infection did not significantly affect the low incidence of autoantibodies to Ro/SSA and La/SSB Ags in NZM2328 mice. However, reactivities to other salivary and lacrimal gland proteins were readily detected. MCMV infection did not significantly alter the spontaneous onset of kidney disease in NZM2328. Thus, chronic inflammation induced by MCMV with decreased secretory function in NZM2328 mice resembles the disease manifestations of human Sjögren’s syndrome. The Journal of Immunology, 2006, 177: 7391–7397.
glands when virus was undetectable by PCR. Other features of human SS, including salivary gland dysfunction and autoantibody production, were investigated to evaluate MCMV infection of NZM2328 mice as a novel model for SS.

Materials and Methods

Mice

Female C57BL/6 (B6) and B6.MRL-Fas<sup>lpr</sup> (B6-lpr) mice were obtained from The Jackson Laboratory. NZM2328 breeders are bred and maintained at the University of Virginia under specific pathogen-free conditions. All animal studies were approved by and conducted in accordance with Animal Care and Use Committee oversight.

MCMV and virus assays

MCMV (Smith Strain; American Type Culture Collection) stock 1 was passaged twice in 6-wk-old BALB/c females and titered on murine embryonic fibroblasts (MEFs). MCMV stock 2 was serially passaged in weanling BALB/c mice and titered on 3T3 cell monolayers. Experimental mice (8–12 wk) were i.p. infected with MCMV in 0.2 ml of sterile PBS (10<sup>5</sup> PFU of stock 1, 10<sup>6</sup> PFU of stock 2). Submandibular plus sublingual gland (SG) homogenate virus titers were determined by standard plaque assay on MEFs as described (8). Salivary and lacrimal gland virus levels were determined by quantitative real-time PCR (QPCR) as described previously (8). All sample measurements were performed in triplicate. Results are reported as the number of MCMV genomes detected per number of endogenous (β-actin) genomes.

Clinical and histological assessment

Submandibular, parotid, sublingual, and lacrimal glands were fixed with 10% phosphate-buffered formaldehyde. Paraflin-embedded tissue sections (5 μm) were stained with H&E or Trichrome in the University of Virginia Research Histology Core. Glandular inflammation in one representative section from each mouse was assessed by the following criteria: 1) diffuse infiltration of mixed cell types (0 = no inflammatory cell infiltration, 1 = diffuse inflammatory cell infiltration <2,500/mm<sup>2</sup>, 2 = diffuse inflammatory cell infiltration <10,000/mm<sup>2</sup>, and 3 = diffuse inflammatory cell infiltration >10,000/mm<sup>2</sup>); and 2) mononuclear cell infiltration in periductal areas (0 = no focal mononuclear cell infiltration around ducts, 1 = one periductal mononuclear cell focus/4 mm<sup>2</sup>, 2 = two periductal mononuclear cell foci/4 mm<sup>2</sup>, 3 = more than three periductal mononuclear cell foci/4 mm<sup>2</sup>, and 4 = confluent infiltration with glandular destruction). Tissue histologies were scored blind by Y. Ohyama and V. A. Carroll. Selected tissue sections were similarly evaluated by Dr. H. Bagavant (Division of Rheumatology and Immunology, University of Virginia, Charlottesville, VA) and Dr. K. Tung (Department of Pathology, University of Virginia). Screening for severe proteinuria (≥300 mg/dl on consecutive screens) in female NZM2328 mice (≥8 wk) was performed biweekly with Multistix 10 SG (Bayer).

Salivary gland functional assessment

Salivary secretion was measured after induction by i.p. injection of pilocarpine (0.5 mg/kg body weight; MP Biomedicals) as unanesthetized mice essentially as described (9). Briefly, saliva was collected continuously from the oral cavity by vacuum suction from 2 to 27 min after pilocarpine induction. This time period coincides with the peak response. Saliva volumes were measured by micropipette and expressed as total volume saliva produced in 25 min.

ELISAs and Western blotting

Anti-Ro and anti-La autoantibodies were measured by ELISA using recombinant murine Ro60, Ro52, and La absorbed onto Immulon-4 (Dynatech) as described previously (10). Western blot analysis was also performed as described previously (10). Briefly, salivary and lacrimal gland extracts from uninfected adult mice were used as Western blot substrates for sera collected from MCMV-infected or control mice.

Results

Kinetics of MCMV clearance in the salivary and lacrimal glands

Delayed MCMV clearance in the salivary gland of B6-lpr mice compared with B6 has been reported (6). We sought to determine the kinetics of viral clearance in the autoimmune-prone strain NZM2328. SMG MCMV levels were assessed over a 100-day time course. As shown in Fig. 1, 14–28 days postinfection (dpi)
Acute and chronic inflammatory lesions in NZM2328 after MCMV infection

During acute MCMV infection, when virus is abundant in the SMG, a diffuse mixed leukocytic infiltrate is found throughout the SMG (4). This type of inflammation can be distinguished from chronic inflammatory lesions with large foci (aggregates >50 mononuclear cells) including T and B cells. B6-lpr, but not B6 mice, develop mild focal inflammation at 100 dpi (1–2 foci per lobe of SMG), despite the absence of detectable virus (6). To examine whether the autoimmune prone strain NZM2328 also develops chronic inflammation in the salivary gland upon MCMV infection and whether it was specific to this organ, we examined exocrine glands (submandibular, parotid, sublingual, and lacrimal gland) and kidneys to assess the type and severity of inflammation histologically. The latest time point studied corresponds to almost 6 mo of age, the time at which females of this strain may begin to spontaneously develop glomerulonephritis, mild chronic sialadenitis, and dacryoadenitis.

Table I summarizes the histological findings after MCMV infection in the SMG. Two doses of MCMV were used with similar results. For days 14–28, the period when high titers of infectious MCMV were detected in SMG of all mice, diffuse infiltration of mixed inflammatory cell types was observed in all groups. The most severe infiltrations were seen in the SMG of female NZM2328 at days 14 and 28, such that glandular epithelium was not visible (Fig. 2). Diffuse infiltration of inflammatory cells was much milder in the SMG of male NZM2328 and female B6-lpr. The acute infiltration decreased at day 56 and was no longer apparent by day 75 in all groups. The presence of diffuse infiltrates correlated with the presence of virus. Acute inflammation was fairly restricted to the SMG, because slight infiltration (histological score 1) was found in the sublingual and parotid glands of all groups for days 14–28 (data not shown).

Interestingly, as diffuse inflammation abated over time in the SMG, MCMV-infected NZM2328 females developed focal mononuclear cell infiltration in the periductal area that was evident at 56 dpi and severe by 75–100 dpi (Fig. 2). Focal inflammation was restricted to the SMG because infiltrates were rarely observed in the mucinous sublingual and serous parotid glands. This is of interest because MCMV prefers to replicate in the mixed acinar epithelium of the SMG. Severe focal inflammation persisted through 200 dpi without detectable virus, whereas spontaneous sialadenitis of uninfected age-matched controls was only mild if even present.

### Materials and Methods

#### Acute and Chronic Inflammation after MCMV Infection

NZM2328 female mice were infected with MCMV (10⁶ PFU). At various times postinfection, tissues were formalin-fixed, sectioned, and stained with H&E. Note the diffuse SMG infiltrate at day 28 during acute virus infection. This was followed by development of large periductal foci in both the SMG and lacrimal gland at days 56 and 100 during viral latency. Uninfected mice were age matched to day 100, corresponding to 5.5 mo of age. Images shown at ×100.
cells over B220/H11001 were seen in B6-lpr males were unaffected by MCMV infection, and few infiltrates were than age-matched controls. The lacrimal glands of NZM2328 males developed focal periductal dacryoadenitis that was more se-

found in this exocrine organ. However, at 100 dpi NZM2328 fe-

ence of MCMV at 14 –28 dpi, diffuse inflammation could not be

histological changes (Fig. 2, Table II). Curiously, despite the pres-

ings suggest that MCMV-induced chronic inflammation in the

SMG is dependent on both genetic and sex-linked factors.

To further investigate the consequences of MCMV-induced chronic inflammatory lesions in the salivary gland, we mea-

sured salivation following induction with pilocarpine, a muscarinic receptor agonist. In uninfected NZM2328 control animals, saliva

yields did not change over the course of study (Fig. 5A). Unex-

pectedly, we found that saliva yields were higher in infected ani-

mals than in age- and gender-matched control animals (Fig. 5, B

and D). However, despite having increased pilocarpine-induced salivation during latent virus infection, only MCMV-infected

NZM2328 females progressively lost this function between 90 and 125 days after infection (Fig. 5, B and D). Thus, MCMV infection

in NZM2328 leads to gender-specific differences in inflammatory lesions observed in the exocrine glands that apparently coincide with the onset of salivary gland dysfunction.

Production of autoantibodies in MCMV-infected NZM2328

In addition to salivary gland dysfunction, SS patients often display autoantibodies to Ro/SSA and La/SSB Ags. MCMV infection did not lead to elevated levels of anti-Ro60, anti-Ro52, or anti-La/SSB autoantibodies in NZM2328 or B6-lpr (Fig. 6). Because autoanti-

tibodies to salivary and lacrimal gland Ags such as anti-muscarinic receptor 3 have also been documented in SS (12), we examined whether organ-targeted autoantibodies develop in MCMV-infected NZM2328. As shown in Fig. 7, autoantibodies recognizing exo-

crine gland Ags are apparent in infected NZM2328 females. In

<table>
<thead>
<tr>
<th>Days Postinfection</th>
<th>Control (100)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>10^5 PFU MCMV stock 2</td>
</tr>
<tr>
<td></td>
<td>NZM2328 F</td>
</tr>
<tr>
<td>n</td>
<td>3 6 12 4</td>
</tr>
<tr>
<td>Acute-diffuse</td>
<td>0 0 0 0</td>
</tr>
<tr>
<td>Chronic-periductal</td>
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</tr>
<tr>
<td></td>
<td>NZM2328 M</td>
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<tr>
<td>n</td>
<td>4 3 9 1</td>
</tr>
<tr>
<td>Acute-diffuse</td>
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Changes in the composition of the mononuclear cell infiltrate over the course of MCMV infection

To characterize the cell populations associated with the acute and chronic infiltrate, SMG were obtained from NZM2328 females at different times following MCMV infection and analyzed by immunohistochemistry (Fig. 3). At day 28 after MCMV infection, when high titers of infectious MCMV were detected, diffuse infiltration of mixed inflammatory cells including Thy1.2+, CD4+, CD8+, and B220+ cells were observed throughout the SMG. In contrast, at day 100 after MCMV infection, when MCMV became undetectable, mononuclear cells infiltrated around ductal structures in defined areas and CD8+ cells were not found. CD4+ and B220+ cells segregated in separate areas and a predominance of CD4+ T cells over B220+ cells was generally observed. These immuno-

histochemical findings are similar to those of SS (11).

Focal sialadenitis was accompanied by interstitial fibrosis and ductal destruction evident by Trichrome staining (Fig. 4). In con-

trast to an uninfected age-matched control gland, blue-staining col-

lagen surrounding the ducts indicative of basement membrane structure was missing in NZM2328 females 100 dpi. In addition, apoptosis of lymphocytes and SMG ductal epithelium was de-

ected 100 dpi by TUNEL staining that was not found in an un-

infected age-matched control gland (data not shown). These his-

tological findings suggest that previous MCMV infection of

NZM2328 females compromises the structural integrity and homeostasis of the SMG after 100 dpi.

Loss of saliva production in MCMV-infected NZM2328 with chronic inflammatory lesions

To further investigate the consequences of MCMV-induced chronic inflammatory lesions within the salivary gland, we mea-
addition, we could immunoprecipitate three distinct protein bands from a human salivary gland cell line extract with one of these sera that were not similarly immunoprecipitated using control sera (Y. Ohyama and S. M. Fu, unpublished data). Taken together, these data substantiate a role for viral induction of autoantibodies to glandular Ags.

Discussion
In this study, we show that NZM2328 lymphocytes respond to acute MCMV infection of the exocrine glands with similar kinetics and vigor as for other strains. However, NZM2328 female mice were distinguished by severe acute and chronic inflammation in the salivary and lacrimal glands. Focal inflammation persisted for at least 200 days without detectable replicating virus. This phenotype is not a general feature of MCMV-induced pathogenesis because B6 mice do not develop such lesions (6). As previously shown, we also noted delayed SMG viral clearance and mild chronic infiltrates in MCMV-infected B6-lpr because Fas may play a role in apoptosis of infected glandular epithelium and Fas-mediated cell death of lymphocytes is important for the down-regulation of immune responses. Although immunocompetent NZM2328 mice are genetically prone to develop spontaneous lupus-like disease including anti-dsDNA autoantibodies and acute and chronic glomerulonephritis, MCMV did not accelerate the incidence of severe proteinuria in this study (V. A. Carroll, H. Baganvat, and M. G. Brown, unpublished data), suggesting that MCMV-induced pathology in the salivary and lacrimal glands does not accelerate overt autoimmune disease in general. Furthermore, MCMV failed to elicit anti-Ro60 or anti-La autoantibodies. Instead, autoantibodies with specificity for salivary and lacrimal gland Ags were detected in latently infected NZM2328 females when focal inflammation in the exocrine glands was severe. Though the target Ags have yet to be identified, we did not observe significant reactivity with proteins in the size range of /H9251-fodrin (120 kDa), a previously discovered target Ag in SS. Correspondingly, a progressive loss in salivary gland function was also observed in NZM2328 females during latent infection. Interestingly, salivary gland function was enhanced by 90 dpi in NZM2328. This phenomenon remains to be explained but may be due to epithelial regeneration after infection. Taken together, MCMV infection of NZM2328 females displays various features of human SS.

Interestingly, whereas MCMV is cleared from systemic organs after 1–2 wk, it persists in the SMG for 6–10 wk depending on viral dose and mouse strain. Recent work indicates viral interference with Ag presentation to CD8+ T cells contributes to viral persistence of this organ (13). CD4+ lymphocytes have a key role in restraining MCMV (14), and salivary gland derived virus is more virulent than that derived from other organs or grown in vitro (15). Therefore, the SMG provides an important niche for efficient MCMV replication, dissemination, and evasion of host immune responses. MCMV tropism and relative persistence in the SMG may be important for induction of chronic inflammation in this organ in NZM2328 after virus is cleared.
We also noted focal dacryoadenitis in latently infected NZM2328 females. The lacrimal and salivary gland both drain to the cervical lymph nodes, which were enlarged in many of the affected animals (V. A. Carroll and M. G. Brown, unpublished observations). There are several potential explanations for the organ-specific infiltrates. Because both of the exocrine glands become infected during early times, CD4/CD25 regulatory T cells specific for viral peptide may accumulate to control sporadic reactivation from latency in the target organ. Low level transcription and translation of viral gene products may be sufficient for activation and retention of lymphocytes in the tissue. In this case, the dysregulation of the immune response inherent to NZM2328 would be responsible for the chronic inflammation observed because B6, a nonautoimmune prone strain, is protected. The nature of immune dysregulation in NZM2328 is under investigation by other groups. Of note is the finding that NZM2328 do not possess a global defect in CD4+/CD25+ regulatory T cells, however, they may require a higher threshold for suppression of particular d3tx-induced autoimmune diseases, namely glomerulonephritis and sialadenitis but not dacryoadenitis, thyroiditis, or prostatitis (16). In contrast, some NZM2328 mice spontaneously develop mild infiltrates into the submandibular and lacrimal glands after 7 mo of age. Therefore, NZM2328 may possess an intrinsic potential for autoimmune lymphocytes specific for glandular self-Ag, and viral infection may activate these cells perhaps through massive glandular destruction during acute infection. A third possibility is molecular mimicry (17), where the MCMV response cross-reacts with glandular Ags. Each of these scenarios is consistent with a genetically programmed failure to control immune responses in the salivary and lacrimal glands, whether to foreign or self-Ag. The specificity of the infiltrating cells in our model remains an open question, and a more detailed study of the SMG-infiltrating lymphocytes is important to delineate these possibilities.

In summary, we describe the development of focal inflammation in the submandibular and lacrimal glands upon MCMV infection of NZM2328 female mice. Furthermore, severe focal inflammation was accompanied by production of autoantibodies against glandular Ags and a progressive loss of salivary gland function. These manifestations support MCMV infection of NZM2328 as a novel virus-induced model for human SS. Viral infection and autoimmune disease have long been associated, however, the mechanism remains elusive. Our model offers a novel system to study the intricacies of the relationship between virus and host, and highlights the interplay of virus and host factors in development of organ-specific chronic inflammation.

**Acknowledgments**
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**Disclosures**
The authors have no financial conflict of interest.
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