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The Impact of Primary and Persistent Cytomegalovirus Infection on the Progression of Acute Colitis in a Murine Model

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Abstract

Cytomegalovirus (CMV) infects 60-100% of the population worldwide. CMV has been implicated in many diseases through the induction of inflammation. Inflammatory bowel disease (IBD) affects over 1 million Americans annually. IBD, in particular ulcerative colitis, has been associated with CMV infection. Here we use a murine model to test if both primary and persistent CMV infections exacerbate colitis. C57Bl/6J mice were injected with mock inoculum or murine CMV (mCMV) 4d (primary infection) or 6wks (persistent infection) before inducing colitis. Colitis was induced by administering 3% DSS (dextran sodium sulfate) in the drinking water for 6 days. Distilled water was given to controls. Disease activity index (DAI), derived from scores for stool consistency, body weight loss, occult blood, and rectal bleeding, was recorded daily. DAI increased early with DSS treatment in mocks when compared with water-treated controls. This was accelerated by both primary and persistent mCMV and appeared to be primarily due to the earlier appearance of gross bleeding versus their mock controls. Mocks reached similar DAI values by day 6. Myeloperoxidase was modestly elevated in the mCMV 4d-DSS over the mock 4d-DSS, however there was no such synergism in the 6 wk groups. Histology was comparable in mock and mCMV groups. Taken together our findings show that mCMV accelerated the development of acute colitis although a milder model of colitis may be needed to better delineate the impact of the virus on disease progression. Further work focusing on disruption of barrier function and bleeding may help determine the underlying mechanisms.

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Keywords

ulcerative colitis; dextran sodium sulfate; cytomegalovirus; inflammation; gross bleeding; disease activity index

Introduction

Inflammatory Bowel Disease (IBD) is a general term for autoimmune diseases of the intestines, which are characterized by chronic or recurrent inflammation. There are two main types of IBD, ulcerative colitis (UC) and Crohn's disease (CD). It is estimated that 1-1.3 million people are affected by IBD in the United States with peak onset ranging in age from 15-30 [1, 2]. CD can affect any part of the gastrointestinal tract. Inflammation is transmural and discontinuous within the intestinal wall and surrounded by healthy intestinal tissue. Occurrence rates of CD has been increasing worldwide particularly in developing countries; however, it is more common in the developed world [3]. UC is limited to the colon and only affects the inner mucosal layer. The disease is intermittent with periods of mild or no symptoms, interspersed with periods where symptoms are more severe. UC has a higher prevalence (7.6 to 246 cases per 100,000 persons per year) than CD (0.03 to 15.6 cases per 100,000 persons per year). Similar to CD, UC incidence rates are higher in developed countries particularly those in Northern Europe and North America [4]. This is due in part to the western lifestyle and diet [5].

The prevailing understanding of IBD initiation is that host genetic factors and environmental factors leads to immune cell activation (i.e. T-cells, mucosal dendritic cells) against normal microbial flora of the intestines resulting in the development of chronic colonic inflammation. The primary causative agent has not been identified; however, many possibilities are being investigated including *Yersinia enterocolitica* [6] and *Mycobacterium paratuberculosis* [7, 8]. The establishment of chronic inflammation can result in the recruitment and activation of other pathogens which can result in exacerbation of IBD through the upregulation of NF- κ B and inflammatory cytokines and chemokines. This includes human cytomegalovirus (HCMV), which is associated with exacerbated IBD [9, 10].

HCMV is a β -herpesvirus that infects a majority of the population worldwide. Infection occurs primarily during childhood and the virus establishes a lifelong persistent infection. In addition, HCMV can infect almost every organ system in the body including the intestines. 70% of patients with CD are seropositive for CMV although less than 5% of tissue and stool samples are CMV positive [11, 12]. Evidence for a role of this virus in CD has been limited. However, the link between HCMV and UC is stronger [13-18]. The virus is prevalent in tissues of these patients [19] and reactivation of the virus in UC patients is associated with steroid-resistance during treatment [16]. The exact role of CMV in IBD is unknown. This virus may be recruited to inflamed intestinal tissue through the recruitment of immune cells (i.e. monocytes), and once present in the inflamed bowels will become reactivated due to inflammatory signaling (TNF- α , IFN- γ). Reactivated virus will in turn activate pro-inflammatory pathways and may also increase the permeability of the gut [20, 21]. While

murine CMV has been shown to exacerbate colitis in persistent/latent models of infection, there has been no side-by-side comparison of the impact of CMV on colitis during primary and persistent phases of infection.

This study sought to determine whether both primary and persistent CMV infection exacerbates colitis, so that it could be determined in future work if underlying mechanisms differ during these phases. We used the murine model of dextran sodium sulfate (DSS)-induced colitis, which results in acute colitis characterized by loose stool, bleeding, and weight loss. This is due to epithelial injury and a robust inflammatory response in the colon [22]. We induced colitis during either the primary or persistent murine CMV (mCMV) infection period to determine if we could detect worse disease score and histological changes.

Materials, Methods & Techniques

Wildtype C57BL/6J mice were obtained from Jackson Laboratories (Bar Harbor, ME, USA). Mice (3-5wks old) were infected with mock inoculum or mCMV (Smith strain) at 3×10^4 PFU i.p. as described previously [23]. These mice were split into 2 cohorts based on time post-inoculation (p.i.) when colitis was induced: 4 d=primary infection, or 6 wk=persistent infection. To induce colitis, mice were given 3% DSS (molecular weight, 40kDa; ICN Biomedicals, Aurora, OH, USA) in distilled water as their drinking water (*ad libitum*), while distilled water was given to control mice. Treatments were given for 6 days. n=4 and 3 in the Mock 4d+H₂O and Mock 6wk+H₂O groups, and 6-8/grp in the other groups. Animal handling procedures were approved by the LSU Health Institutional Animal Care and Use Committee and were in accordance with the Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health.

A previously validated disease activity index (DAI) monitoring was performed daily beginning on day 0 (just prior to DSS treatment) and continued through 6 days of DSS treatment. Several parameters were included in the assessment: stool (normal = 0; loose = 2; diarrhea = 4), occult blood as determined by a guaiac paper test (ColoScreen; Helena Laboratories Inc., Beaumont, TX, USA [24] (negative = 0; positive =2), gross blood (negative = 0, positive = 4) and weight change (compared the original body weight: <1% = 0; 1-5% = 1; 5-10% = 2; 10-15% = 3 and >15% = 4) [25].

At day 6, mice were weighted, and anesthetized with ketamine and xylazine i.p. Colons were harvested. In the 6 wk group, the harvested colons were weighed, and the colon length was measured. In all mice, samples of colon were placed in formalin for histology. The histological score was determined by a blinded observer using a previously published method [26], taking into account severity of inflammation, extent of injury, as well as crypt damage. The maximum possible score was 40.

A piece of colon was also taken for measurement of myeloperoxidase (MPO), as an indicator of inflammatory infiltrate. Colonic tissue was cleaned, weighed, and snap frozen for storage at -80°C until subsequent analysis. The o-dianisidine method [27] was used for

measurement of MPO activity and output was expressed as the amount of enzyme necessary to produce a change in absorbance of 1.0 unit per minute per gram of tissue (U/g tissue).

Statistical Analysis

All values are reported as mean $\pm$ SEM. ANOVAs were performed using Fisher's post hoc test to compare experimental groups with a statistical significance set at $p = 0.05$.

Results

Primary mCMV Infection

During primary infection (DSS administered at 4 days p.i.), the DAI of both mock and mCMV DSS groups were significantly increased compared to controls (Fig. 1A), with the mCMV group rising slightly earlier. The DAI of the mCMV 4d+DSS group was significantly increased over Mock 4d+DSS mice on day 5. The difference in DAI between the colitic mock and mCMV groups was primarily due to gross bleeding, which was evident in both DSS groups on days 5 and 6 but significantly higher in mCMV-infected mice (Fig. 1B).

In the primary infection cohort, colons were taken for histology after 6 days DSS, and histological scores were elevated. Figure 2 shows that both DSS groups showed elevated scores in all categories, and while there was a tendency for the mCMV group to exhibit higher severity of inflammation this was not significant, and the mCMV 4d+DSS group was comparable to the Mock 4d+DSS group. All H₂O-treated mice had scores of zero.

The levels of MPO measured in tissues of DSS mice were significantly higher than those in water groups (Fig. 3) in the 4 day p.i. cohort. Surprisingly, the MPO in the mCMV 4d+H₂O group was slightly lower than mock counterparts, and MPO increased 2.7-fold with DSS. In contrast the mock 4d+DSS mice exhibited a more modest elevation of 35% over their controls. Thus, like the severity of inflammation score in the histology, there was a tendency for higher MPO in the mCMV 4d+DSS group over Mock counterparts but this did not reach significance.

The percent body weight change was slightly (but not significantly) decreased in both mock and mCMV DSS groups when compared to corresponding control groups in the 4 day p.i. cohort (Table 1).

Persistent mCMV Infection

Persistent CMV infection was also investigated since many patients become infected during childhood, prior to the onset of IBD. In this study, mice were infected with CMV for 6 weeks prior to DSS treatment. The DAI for both DSS groups was elevated from day 1 onwards when compared to corresponding water controls (Fig. 4A). In the DSS groups, CMV infection led to a significantly higher DAI than the Mock 6wk-DSS group on day 4. Gross bleeding in mock 6wk+DSS group was evident on days 5 and 6 (Fig. 4B), as also shown for the mock 4d cohort. However, gross bleeding was evident a day earlier in persistently infected mCMV 6wk+DSS group, corresponding to the higher DAI in these mice. By day 6, the DAI and gross bleeding scores were comparable in the Mock 6wk+DSS

and CMV 6wk+DSS groups. As shown for the primary infection mice, the percent body weight change was slightly, but not significantly, reduced by DSS in the 6 wk groups (Table 1).

Histological scores in all categories were increased in both DSS groups versus H₂O-treated controls, although this did not reach significance for most parameters in the Mock 6wk+DSS group (Fig. 5). While mCMV did not significantly exacerbate the histological changes when compared to the Mock 6wk+DSS group, there was a tendency for the scores to be slightly higher in the mCMV-infected animals.

As expected, the activity of MPO was elevated in the Mock 6wk+DSS mice when compared to their water counterparts (Fig 6). Unlike in the primary infection group, the water-treated mCMV-infected mice exhibited a similar increase in MPO levels (not significant). These factors did not synergize, and the MPO levels in the mCMV 6wk+DSS group were comparable to that in groups with either DSS or mCMV alone.

In addition, in the 6 wk p.i. groups, we measured the colon lengths as an index of colon damage. As expected, colon length decreased in the Mock 6wk+DSS group. This was not altered by mCMV infection (Fig. 7).

Discussion

Cytomegalovirus has been implicated in UC, in particular in severe cases, and in steroid refractive UC. This is likely in part due to increased permeability and enhanced inflammation by the virus [20, 21]. Our data supports a role for CMV infection (both primary and persistent) in colitis. In both primary and persistent mCMV infection, the accelerated development of colitis appeared to be primarily due to an earlier appearance of gross bleeding. The mechanism by which mCMV did this is not known. However, it is plausible that in the primary infection group the accelerated gross bleeding in CMV 4d+DSS group was due to exacerbated inflammatory processes causing earlier damage to the intestinal wall, and thus bleeding, as suggested by the higher MPO levels in these mice. In contrast, in the persistent infection group there was a tendency for increased crypt damage and extent of injury rather than severity of inflammation, which may have contributed to more bleeding in this group. However, the differences in these parameters were modest, perhaps because the colitis develops quite rapidly in this model, and histology and MPO were taken on Day 6, therefore the window during which more pronounced differences may have been detected could have been missed. A milder model of colitis might be better suited to better interrogate the impact of mCMV on these parameters. Nonetheless, our findings suggest that both primary and persistent infection may accelerate colitis, and some focus on the worsened bleeding may help elucidate the underlying mechanisms.

The fact that the overall DAI scores were not exacerbated by mCMV on the final day of treatment may be a reflection of the dose of DSS we chose for this study. The toxicity of DSS on the colon may have been too severe to allow for continuous exacerbation by mCMV. Such a possibility is supported by another study in which the authors used a lower concentration of DSS (2.5%). This caused mild colitis. Furthermore, they used a higher

mCMV inoculation dose given 4 wks before induction of colitis and were able to show that mCMV exacerbated DSS colitis [21]. This was accompanied by increased inflammatory cell infiltrate in the colon, shorter colon length, and decreased body weight change on day 7 in the mCMV-infected mice versus mock counterparts. Furthermore they found that CMV +DSS mice displayed more significant crypt damage compared to controls suggesting that CMV is accelerating and/or exacerbating damage to the colon wall. Although bleeding not examined in that study, increased inflammation and/or damage to the gut wall may help explain the presence of gross bleeding during the development of colitis in our study. The subtle differences between the components of the histology scores in the 4 day and 6 wk cohorts also suggest that the timing of the colitis with respect to CMV infection may be worth further investigation to determine if different mechanisms underlie the exacerbation of disease at different phases of infection.

While we found some differences in the severity of DAI and gross bleeding in mCMV-infected mice versus their mock counterparts our findings did not reveal a robust enough exacerbation of disease to use our model to interrogate mechanisms underlying the ability of CMV to worsen colitis. However, taken along with other studies finding a deleterious impact of mCMV on colitis [9, 11, 13-17, 19-21, 28], we suggest that although small, the changes we detected may be meaningful, and set the stage for future studies into underlying mechanisms, in particular the aspect of enhanced bleeding. Another potentially important consideration in designing future studies is addressing the possible link between steroid-resistant UC and HCMV. It remains controversial whether HCMV is reactivated due to the immunosuppressive effects of steroids, or the virus alters the disease process in such a way as to lead to steroid resistance [14]. While the use of antivirals in patients with evidence of HCMV infection may be useful, the mixed success of this approach, along with the toxic side effects of this drug, suggests there is an urgent need to develop other approaches to address this.

Conclusions

Overall, these findings support the treatment of at least a susceptible subset of IBD patients with antivirals targeting HCMV (i.e. ganciclovir) to reduce the severity of colonic injury. These results underscore the need for further investigation into the mechanisms underlying the impact of different phases of CMV infection on IBD, with some focus on how CMV might affect coagulation pathways and platelet responses [29, 30], and gut wall integrity [20], which are already perturbed during IBD. Less severe, more chronic models of colitis such as recently used by Matsumura et al. [28] will be of benefit to enhance elucidation of the role of CMV in colitis.

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Figure 1.

DAI (panel A) and score for gross bleeding (panel B) for mice injected with mock inoculum or mCMV, and given distilled water or DSS in their drinking water beginning 4 days p.i.

* $P < 0.05$ mCMV 4d+DSS vs. Mock 4d+H₂O; # $p < 0.05$ DSS groups vs. H₂O Controls;

$p < 0.005$ DSS groups vs. H₂O Controls; ^ $p < 0.05$ CMV 4d+DSS vs. Mock 4d+DSS.

Figure 2.

Histological scores of colons from mice exposed to Mock-inoculum or mCMV, and 4 days later exposed to 6 days of DSS. Control Mock and mCMV mice received H₂O, and did not show any score therefore cannot be seen on the figure. # P<0.05 vs. H₂O controls.

Figure 3.

MPO levels in colons from mice exposed to Mock-inoculum or mCMV, and 4 days later exposed to 6 days of DSS. Control Mock and mCMV mice received H₂O. # $P < 0.05$ vs. corresponding H₂O control.

Figure 4.

DAI (panel A) and score for gross bleeding (panel B) for mice injected with mock inoculum or mCMV, and given distilled water or DSS in their drinking water beginning 6 weeks p.i. * $p < 0.05$ Both DSS groups vs. Mock 6wk+H₂O; # $p < 0.01$ DSS groups vs. H₂O Controls; ## $p < 0.005$ DSS groups vs. H₂O Controls; § $P < 0.05$ CMV 4d+DSS vs. all groups; ^ $P < 0.005$ CMV 4d+DSS vs. Mock 4d+DSS; ~ $P = 0.0531$ CMV 4d+DSS vs. Mock 4d+DSS

Figure 5.

Histological scores of colons from mice exposed to Mock-inoculum or mCMV, and at 6 weeks p.i. given 6 days of DSS in their drinking water. Control Mock and mCMV mice received H₂O. # P<0.05 vs. corresponding H₂O control.

Figure 6.

MPO levels in colons from mice exposed to Mock-inoculum or mCMV, and 6 weeks later exposed to 6 days of DSS. Control Mock and mCMV mice received H₂O. * P<0.05 vs. Mock 6wk+H₂O group.

Figure 7.

Colon length, a feature of severe colitis, was determined at 6 days of DSS treatment in mock- and mCMV-inoculated 6 wk mice. H₂O was given to control mice. #P<0.0001 vs. H₂O controls.

Table 1

Body weight expressed as percent of control weight (=100%) at day 0 just before H₂O or DSS treatment. Mice were injected with mock inoculum or mCMV 4d or 6wk prior to beginning the measurements.

Groups	Day 4	Day 5	Day 6
Mock 4d+H ₂ O	101.8±1.58	103.1±0.81	103.5±1.42
Mock 4d+DSS	100.1±1.40	96.0±1.35	90.6±2.15
mCMV 4d+H ₂ O	101.0±1.34	101.7±1.27	101.1±1.22
mCMV 4d+DSS	100.6±0.35	95.9±0.77	90.7±1.94
Mock 6wk+H ₂ O	98.8±1.00	99.6±1.84	101.6±0.71
Mock 6wk+DSS	98.3±1.21	97.3±1.02	92.2±1.10
mCMV 6wk+H ₂ O	99.9±0.42	100.0±0.72	100.0±0.58
mCMV 6wk+DSS	99.2±1.01	97.7±1.12	95.1±2.00