Human Cytomegalovirus Enhances Chemokine Production by Lipopolysaccharide-Stimulated Lamina Propria Macrophages

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To elucidate the role of mucosal macrophages in intestinal human cytomegalovirus (HCMV) disease, primary lamina propria macrophages (LPM) were isolated from normal human jejunum, infected with HCMV, and studied for their cytokine responses. HCMV infection of LPM was confirmed by the presence of HCMV IE72 (UL123), pp65 (UL83), and glycoprotein B (UL55) proteins, which were detected by immunofluorescence, beginning at postinfection (pi) day 3, and were sustained through pi day 12 in 0.1%–0.5% of LPM. The late protein pp28 (UL99) was also detected up to pi day 12, consistent with productive infection. HCMV infection in LPM was characterized by quantitative competitive polymerase chain reaction, with maximum levels of HCMV DNA detected at pi day 7. HCMV infection of the LPM augmented lipopolysaccharide-inducible chemokine (interleukin [IL]–8 and macrophage inflammatory protein–1α) and cytokine (IL–6) production. These findings suggest that mucosal macrophages, via enhanced mediator production, play an important role in intestinal inflammation associated with HCMV infection.

Human cytomegalovirus (HCMV), a β-herpesvirus with a host range restricted to humans [1], infects up to 70% of adults in industrial countries and up to 90% in developing countries [2]. HCMV infection is usually asymptomatic but, in immunosuppressed persons, can cause severe retinitis, esophagitis, colitis, enteritis, and pneumonitis.

Monocytes serve as a reservoir for latent HCMV in vivo [3], apparently becoming infected as monocyte precursor cells in the bone marrow [4–6]. After their release, circulating HCMV-infected monocytes [3, 7] likely disseminate HCMV to organ sites, where immediate early, early, and late viral transcripts have been detected in tissue macrophages [8]. The presence of HCMV-infected macrophages in infected tissues raises the possibility that these cells contribute to HCMV-associated organ pathology, particularly in the gastrointestinal tract mucosa, the largest reservoir of macrophages in the body [9] and an important target of HCMV infection [10, 11].

HCMV nucleic acids have been identified in all major leukocyte populations and in fibroblasts, epithelial cells, endothelial cells, and smooth-muscle cells [12], but high-level replication of virus in vitro is restricted to primary fibroblasts. This restriction is believed to be due to incomplete target cell differentiation in vitro [13]. Monocytes, for example, support HCMV infection after in vitro differentiation [14–16]. HCMV infection of monocytes and macrophages, unlike that of fibroblasts, is transmitted from cell to cell and is not cytolytic [16], yet the virus causes disruption of the microtubule network and Golgi apparatus and causes decreased surface expression of major histocompatibility complex class II molecules [17]. Infection by HCMV alters important monocyte functions, such as the production of tumor necrosis factor (TNF)–α [18] and interleukin (IL)–6 [19]. HCMV also can modulate monocyte-derived macrophages and monocyte-derived cell line production of chemokines, including the CC or β-chemokine macrophage inflammatory protein (MIP)–1α [20], MIP-1β [20, 21], monocyte chemoattractant protein–1 [20, 21], and RANTES [21] and the CXC or α-chemokine IL–8 [22, 23].

Since intestinal macrophages have phenotypic and functional features that distinguish them from blood monocytes [24–26], we investigated the permissiveness of human primary lamina propria macrophages (LPM) for productive HCMV infection and assessed the ability of HCMV to alter chemokine and cytokine production by these important tissue macrophages. Here, we describe the in vitro infection of terminally differentiated primary human LPM by a clinical isolate of HCMV, to determine whether such infection enhances inducible production of the chemokines IL–8 and MIP–1α and the cytokine IL–6, potent mediators of inflammation.
Materials and Methods

Intestinal tissue biopsies. Biopsy specimens of small intestinal tissue were obtained from AIDS patients with HCMV enteritis. Confirmation of infection was based on the gross appearance of mucosal inflammation, histopathology showing cytomegalic inclusion cells, and infiltration of inflammatory cells.

Isolation of LPM. LPM were isolated, as described elsewhere [24]. In brief, segments of healthy jejunum were obtained from donors without known local or systemic infection or immunosuppressive disease who were undergoing gastrojejunostomy for obesity. After dissection of the segment at the muscularis propria, the mucosa was treated with 200 μg/mL of dithiothreitol (Sigma Chemical), 0.2 M EDTA (Fisher Scientific), and 10 mM 2-mercaptoethanol (Sigma) to remove the epithelium. The remaining tissue (lamina propria) was minced into 0.5-mm³ pieces and was digested twice (45 min at 37°C) with a neutral protease (Dispase [75 μg/mL], grade I, specific activity > 6 U/mg; Boehringer Mannheim). The lamina propria mononuclear cells (lymphocytes and macrophages) were separated by density gradient sedimentation and then were subjected to counterflow centrifugal elutriation to purify the macrophages [27]. The cells isolated by this procedure contained <1% CD3⁺ lymphocytes and displayed the phenotype, morphologic features, ultrastructure, and phagocytic activity of macrophages [24]. LPM were cultured at a concentration of 3 × 10⁶ cells/mL in complete RPMI 1640 medium containing 10% fetal calf serum (FCS; Gibco) and 2 mM l-glutamine, penicillin (100 U/mL), streptomycin (100 μg/mL), and gentamicin (50 μg/mL; all from Mediatech).

Preparation of HCMV inoculum and infection of LPM. A recent HCV clinical isolate (Powers; obtained from J. Nelson, Oregon Health Sciences University) [28] was passaged through primary human foreskin fibroblasts and cultured in complete medium 199 (Mediatech), supplemented, as described above, with ciprofloxacin (15 μg/mL; Gibco). Culture supernatants were shown to be free of mycoplasma by polymerase chain reaction (PCR) and a mycoplasma detection kit (American Type Tissue Collection) and then were frozen at −80°C. Freshly isolated LPM were cultured 48 h in complete RPMI 1640 medium and then were inoculated with stock HCMV at an MOI of 0.5–1 pfu/cell. Parallel cultures of LPM were mock inoculated with complete medium 199. Cells were incubated with virus for 2 h, after which the LPM, which are nonadherent [24], were washed twice with sterile PBS, resuspended in complete RPMI 1640, and plated in 6-well tissue culture plates (Becton Dickinson Labware; 6 × 10⁶ cells/3 mL/well). Infection and chemokine-cytokine production were assessed on postinfection (pi) days 3, 5, 7, and 12. The viability (always >92%) and the number of LPM at each time point were equivalent, as determined by propidium iodide exclusion and protein quantitation with a protein assay reagent (Coomassie Plus; Pierce), respectively. We added lipopolysaccharide (LPS; 10 μg/mL; Sigma) to designated cultures on pi day 5.

ELISA for soluble mediators. Culture supernatants were tested by ELISA for IL-6, MIP-1α, and IL-8 (R&D Systems; sensitivities of <0.7, <7, and 10 pg/mL, respectively). We determined the significance of the difference in mean cytokine levels in culture supernatants from inoculated and mock-inoculated cultures by analysis of variance with StatView 4.5 software (Abacus Concepts).

Indirect IFA for HCMV proteins. On pi days 3, 5, 7, and 12, cytospun preparations of LPM were fixed in 3% paraformaldehyde for 15 min at room temperature (RT) and were permeabilized with 0.0001% saponin (Sigma) in PBS. After brief incubation (15 min at RT) in 2% normal goat serum (Atlanta Biologicals), the cells were incubated (45 min at 37°C) with one of the following mouse monoclonal antibodies specific for HCMV proteins: p63-27 (specific for IE-1, UL123, the major immediate early gene product), 65-8 (specific for pp65, UL83, a major tegument protein), 7-17 plus 58-15 (specific for glycoprotein B [gb], UL55, a major envelope glycoprotein) [29], or 41-18 (specific for pp28, UL99, a late protein in the tegument of the mature virion). The binding of the primary antibody was detected with fluorescein isothiocyanate–conjugated polyclonal goat anti–mouse IgG (Southern Biotechnology Associates) diluted 1:50 in PBS with 1% FCS PCR serum (45 min at 37°C). Control cells were stained with anti–HLA-DR (Becton Dickinson). After the staining, the cells were fixed in 1% paraformaldehyde, counterstained (5 min at RT) with Evans blue (Sigma), and then mounted with an antifade kit (Prolong; Molecular Probes) to reduce quenching. Immunofluorescence of the LPM was visualized with an upright Leitz microscope.

Quantitative-competitive (QC)–PCR assay for HCMV DNA. On pi days 3, 5, 7, and 12, 10⁶ LPM were pelleted in 0.5-μL microfuge tubes and resuspended in 50 μL of buffer containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 2.5 mM MgCl₂, 1 mg/mL gelatin, 0.45% Nonidet P40 (Boehringer Mannheim), 0.45% Tween 20 (Fisher), and 60 μg/mL proteinase K (Boehringer Mannheim) for rapid DNA preparation. The cell suspension was incubated at 56°C for 2 h and then at 95°C for 10 min to inactivate the proteinase K. The resultant cell extracts were stored at −80°C.

To quantitate HCMV DNA, the amount of extract in ~5 × 10⁴ LPM was combined with varying amounts of DNA competitor, and QC-PCR was performed, as described in detail elsewhere [30]. In brief, the primer template for the gene encoding HCMV gb is contained in the competitor pQCPCR.CMV.1. The primer template for the gene encoding glyceraldehyde-3-phosphate-dehydrogenase (G3PDH) is contained in the competitor pQCPCR.GFO1.2 [31]. The 5ʹ sense primer for gb was 5ʹ-ACGTGAAGGAAATCGCCAGGA-3ʹ; and the 3ʹ anti-sense primer was 5ʹ-biotin-AGTTCCAGTACCCTGAAGTC-3ʹ. For G3PDH, the 5ʹ sense primer was 5ʹ-TCCTGACCAACACTG-3ʹ and the 3ʹ anti-sense primer was 5ʹ-biotin-GCCTGCTTCAACACTT-3ʹ. The following conditions were used for amplification: 90°C for 1 min, 55°C for 30 s, and 72°C for 1 min for 35 cycles. PCR products for both gb and G3PDH had a final MgCl₂ concentration of 2.0 mM. The amplified DNA was quantitated by ELISA, as described elsewhere [30]. The detection oligonucleotide for gb was 5ʹ-GCAACACCCGCTAGGAATGTCA-3ʹ; and that for G3PDH was 5ʹ-CTCTGACCTGGCCTAGAAGAACCT-3ʹ. Copies of HCMV DNA relative to genome equivalents (copies of cellular DNA) were expressed as the quotient of the copies of gb divided by the copies of G3PDH.

Results

To elucidate the role of macrophages in the pathogenesis of HCMV-induced intestinal inflammation, we first examined intestinal tissue specimens from patients with HCMV infection and
intestine inflammation for the presence of macrophages. As shown in the representative section of intestinal tissue from an AIDS patient with enteritis (figure 1), cytomegalic inclusion cells pathognomonic for HCMV infection were present, along with macrophages, lymphocytes, and polymorphonuclear cells, in the lamina propria. The close association between HCMV inclusion cells and LPM suggested that LPM participate in the inflammatory response in HCMV gastrointestinal disease.

The permissiveness of LPM to HCMV was determined next, by exposing primary LPM in vitro to a clinical isolate of HCMV and then examining the cells by immunofluorescence for viral proteins. Immediate early, structural, and late HCMV gene products were consistently detected in HCMV-infected LPM on pi days 3, 5, 7, and 12. Infected LPM on pi day 3 expressed the immediate early gene product IE-1 (UL123), the structural protein pp65 (UL83), the envelope glycoprotein gB (UL55), and the late tegument protein pp28 (UL99) (figure 2A–2D, respectively). The cytoplasmic vacuoles observed in pp28-positive cells were similar to those reported for monocyte-derived macrophages [16, 17]. In addition, several cells that displayed HCMV proteins were markedly enlarged, as described for HCMV-infected monocyte-derived macrophages [20]. Of the LPM, 0.1%–0.5% expressed pp65, gB, and pp28, and ~0.1% expressed IE-1. In contrast, uninfected LPM did not display HCMV gene products when stained with the same antibodies, although 50% of LPM stained with anti–HLA-DR antibodies and irrelevant antibody (to murine leukemia virus gp70) routinely showed no staining (data not shown). The addition of LPS at pi day 5 did not alter the number of LPM that expressed HCMV proteins.

To assess the kinetics of HCMV infection, LPM were analyzed by QC-PCR for viral DNA. HCMV DNA was detected in the LPM as early as pi day 3, increased 8-fold by pi day 7, and remained elevated through pi day 12 (figure 3), confirming that the LPM were infected with the virus. Similar to the immunofluorescence findings (figure 2), the addition of LPS at pi day 5 did not significantly change the amount of HCMV DNA in virus-inoculated cultures, suggesting that LPS did not directly influence HCMV replication in these cells.

Since macrophage chemokines and cytokines play a critical role in the recruitment and activation of inflammatory cells, we next determined the effect of HCMV infection on LPM chemokine and cytokine production. Whereas uninfected and HCMV-infected LPM spontaneously released undetectable or low levels of mediators as long as 12 days after infection, LPS stimulation caused both uninfected and HCMV-infected LPM to release substantial levels of IL-8 (figure 4A) and MIP-1α (figure 4B). However, the LPS-stimulated HCMV-infected LPM consistently released significantly more IL-8 (pi days 7 and 12) and MIP-1α (pi day 7) than LPS-stimulated uninfected LPM. In addition, the LPS-stimulated HCMV-infected LPM produced significantly higher levels of IL-6 than the LPS-stimulated uninfected LPM (pi days 7 and 12; figure 4C). Thus, LPM were induced by LPS to produce IL-8, MIP-1α, and IL-6, and this induction was enhanced significantly by HCMV infection.

Figure 1. Histopathology of small intestinal tissue of an AIDS patient with human cytomegalovirus enteritis. Section shows presence of cytomegalic cells containing cytoplasmic inclusions (arrow A) closely associated with macrophages (arrow B) and presence of polymorphonuclear cells (arrow C). Magnification, ×40.
Discussion

Our findings show that HCMV infects primary LPM in vitro. The presence of HCMV DNA and gene products in the cells, shown by immunofluorescence and quantitative PCR, confirmed that at least a subpopulation of intestinal macrophages supported productive HCMV infection. A low number (0.1%–0.5%) of LPM, which are terminally differentiated cells, expressed HCMV proteins, contrasting with the relatively high number (15%) of peripheral blood monocyte–derived macrophages reported to express HCMV proteins, such as IE-1 [16]. The difference in the expression of HCMV gene products by LPM versus monocyte-derived macrophages may be due to the cells’ respective levels of differentiation, as suggested by Nelson et al. [13]. Our findings demonstrate that at least a small percentage of LPM were sufficiently differentiated to support productive HCMV infection.

HCMV infection of unstimulated LPM did not cause production of MIP-1α or IL-6, but HCMV infection followed by LPS stimulation of LPM resulted in MIP-1α and IL-6 production. HCMV infection also significantly enhanced LPS-induced IL-8 production by LPM. These findings are particularly striking, because few cells (≤0.5%) were productively infected, as detected by monoclonal antibodies specific for virion structural proteins. We have not yet determined whether the HCMV-infected cells were responsible for the increased levels of IL-8 or whether HCMV-infected cells acted on nearby uninfected LPM to augment IL-8 production.

The levels of IL-8 produced by primary LPM in our study were similar to those that induced HCMV replication in a human

Figure 2. Indirect immunofluorescence analysis of lamina propria macrophages (LPM) for human cytomegalovirus (HCMV) proteins. LPM were exposed to HCMV and were examined 3 days later for IE-1, the major immediate-early gene product (A); pp65 (UL83), a major tegument protein (B); glycoprotein B (UL55), a major envelope glycoprotein (C); and pp28 (UL99), a late protein present in the tegument of the mature virion (D). Magnification, ×40.

Figure 3. Kinetics of human cytomegalovirus (HCMV) DNA production in lamina propria macrophages (LPM). LPM exposed to HCMV (arrow) were treated 5 days later with lipopolysaccharide (LPS; 10 μg/mL) for 24 h. Results are no. of copies of HCMV DNA (glycoprotein B) relative to no. of copies of cellular DNA (glyceraldehyde-3-phosphate-dehydrogenase) in LPM from 3 separate donors, assayed in duplicate. No copies of HCMV DNA were detected in LPM or LPM + LPS (data are mean ± SEM of 6 values).
lung fibroblast cell line [32]. The ability of IL-8 to induce HCMV replication could be accomplished through cytokine-stimulated transcription of HCMV late genes before DNA replication [33] and activation of early events in HCMV replication [34]. Together, these findings have clinical relevance to AIDS, since IL-8 production is increased in AIDS patients, possibly due to human immunodeficiency virus type 1 (HIV-1)–mediated activation of NF-κB and NF–IL-6 [35]. Thus, HIV-1–induced IL-8 could, in turn, promote HCMV replication in intestinal LPM in persons coinfected with both HIV-1 and HCMV.

IL-8 may contribute to the pathogenesis of HCMV-associated inflammation not only by enhancing HCMV replication, as described above, but also by recruiting and activating neutrophils at sites of infection [36]. Neutrophil recruitment could be further enhanced by the α-chemokine vCXC-1, which is encoded by UL146 of HCMV [37]. Recruited and activated neutrophils might also become infected and disseminate virus to distal tissues. These activities give IL-8 the capacity to play an important role in the pathogenesis of the gastrointestinal inflammation associated with HCMV.

HCMV infection significantly elevated MIP-1α production by LPS-stimulated LPM, albeit transiently. The effect of LPS stimulation may have masked HCMV-augmented MIP-1α production at the later time point. MIP-1α, a CC or β-chemokine with chemotactic activity for monocytes and lymphocytes [38], may contribute to HCMV-associated inflammation by increasing recruitment of mononuclear cells to sites of HCMV replication. MIP-1α also inhibits stem cell hematopoiesis [39] and, along with IL-8, suppresses myeloid progenitor cell colony formation [40], thereby providing a mechanism, at least in part, for the immunosuppression associated with HCMV disease.

IL-6 was also induced by HCMV infection of LPM stimulated with LPS. This pluripotent cytokine mediates B cell growth and differentiation, T cell activation, acute phase reactions, and antibody responses [41–43] and has potent anti-inflammatory activity via its ability to down-regulate the production of IL-1β and TNF-α [44, 45]. The release of IL-6 by HCMV-infected LPM suggests that this cytokine also is involved in the gastrointestinal disease associated with HCMV infection, analogous to the release of IL-6 by HCMV-infected microglia, which has been proposed as a mechanism for HCMV-associated neuroinflammation in the brain [46]. Thus, the production of ≥3 inflammatory mediators is enhanced by HCMV infection of LPM. Together, these cytokines could initiate or induce the inflammatory processes that characterize end-organ diseases such as HCMV colitis.

Figure 4. Kinetics of interleukin (IL)–8, macrophage inflammatory protein (MIP)–1α, and IL-6 production by human cytomegalovirus (HCMV)–exposed and –unexposed lamina propria macrophages (LPM) treated with lipopolysaccharide (LPS; 10 μg/mL) 5 days after infection (arrows). Results are mean pg/mL ± SEM of (A) IL-8, (B) MIP-1α, and (C) IL-6 produced by LPM from 3 separate donors, assayed in triplicate. *Amount of cytokine produced by HCMV-infected LPM stimulated with LPS (LPM+HCMV+LPS) significantly exceeded the amount produced by uninfected LPM stimulated with LPS (LPM+LPS) for IL-8 (P < .0001), MIP-1α (P < .0001), and IL-6 (P = .003). Results are representative of 3 separate experiments.

Because LPM do not express surface CD14 [24, 25], the receptor for complexes of LPS and LPS-binding protein, the ability of LPS to induce LPM to produce IL-8, MIP-1α, and IL-6 suggests that an alternate, CD14-independent LPS signaling pathway may operate in LPM. Indeed, CD14 is not an absolute requirement for LPS activation of mononuclear phagocytes [47], LPM express toll-like receptors 2 and 4 [25]. It remains to be determined whether other mechanisms of LPS signaling exist.

In summary, the data presented here indicate that resident intestinal LPM are permissive to a clinical isolate of HCMV and that HCMV infection primes LPM for production of the chemokines IL-8 and MIP-1α and the cytokine IL-6. These findings suggest that LPM may contribute to the inflammation associ-
ated with HCMV disease through the production of these potent inflammatory mediators.

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References