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Research Article

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Cytomegalovirus Induction of Tumor Necrosis Factor-α by Human Monocytes and Mucosal Macrophages

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Abstract

Cytomegalovirus (CMV) is a major cause of inflammatory organ disease in immunosuppressed persons. To elucidate the mechanisms of CMV-induced inflammation, we investigated whether tumor necrosis factor-α (TNF-α) was involved in the pathogenesis of CMV colitis in patients with AIDS. In in situ hybridization experiments, TNF-α mRNA was shown to be abundantly present in colonic mucosa from AIDS patients with CMV colitis but not in colonic mucosa from control (AIDS and normal) subjects. The TNF-α transcripts, identified in macrophage-like cells containing cytomegalic inclusions, were positively associated with CMV, but not HIV-1, within the mucosa. In in vitro experiments, a patient-derived isolate of CMV, but not HIV-1_{Ba-L}, induced human monocytes to express TNF-α mRNA and to release increased levels of TNF-α peptide following stimulation. CMV induction of TNF-α may play a critical role in CMV-induced inflammation and, since TNF-α upregulates expression of HIV-1, offers a mechanism by which CMV could serve as a co-factor for HIV-1 expression without both viruses infecting the same cell. (*J. Clin. Invest.* 1992. 90:1642–1648.) Key words: cytomegalovirus • tumor necrosis factor • AIDS • HIV

Introduction

Cytomegalovirus (CMV)[1] infection in immunosuppressed persons can lead to severe colitis, retinitis, and pneumonitis. Features of these inflammatory reactions, irrespective of the organ involved, include the presence of viral inclusion cells, accumulation of mononuclear inflammatory cells, and tissue necrosis. These shared features suggest a common pathogenesis involving the migration of circulating mononuclear cells, latently infected with CMV (1–3), to tissue sites. In AIDS, an immunosuppressive disease associated with CMV viremia and tissue inflammation (4, 5), CMV has been suggested to function as a co-factor (6), possibly by its ability to upregulate the expression of HIV-1 (7–9). Since CMV can infect monocytes and since monocytes are a source of tumor necrosis factor-α (TNF-α), a product capable of promoting both tissue inflammation and HIV-1 expression (10, 11), we investigated whether CMV could induce human monocytes/macrophages to produce TNF-α.

Methods

Colon tissue specimens. Colonoscopic biopsies were obtained from four HIV-infected homosexual males with AIDS and CMV colitis. Diagnosis of CMV colitis was based on the presence of persistent diarrhea, endoscopic visualization of mucosal inflammation and ulceration, histopathology showing cytomegalic inclusion cells and inflammation with or without vasculitis, and isolation of CMV from a biopsy specimen on human diploid lung fibroblasts (MRC-5, American Type Culture Collection, Rockville, MD). Control colon specimens were obtained at colonoscopy from four homosexual males with AIDS and a healthy seronegative subject without gastrointestinal symptoms with histologically normal colonic mucosa. For patients and control subjects, informed consent was obtained before colonoscopy and biopsy.

In situ hybridization. Tissue sections (6–8 μm) were fixed with periodate-lysine-paraformaldehyde-glutaraldehyde onto silanized slides and hybridized as described (12–14). After deparaffinization of the tissue sections with xylene, the sections were permeabilized with proteinase K (100 μg/ml for tissue, 10 μg/ml for cells), acetylated with 0.25% acetic anhydride in 0.1 M triethanolamine buffer (pH 8.0), and rehydrated in graded ethanols. After prehybridization in 50% formamide-1× Denhardt's solution-2× SSC-50 mM Tris-1 mM EDTA-0.5% mg/ml tRNA for 3 h at 40°C, the specimens were incubated with hybridization mixture containing the ^{35}S-labeled cDNA probe (5×10^5 cpm/slide) encoding human TNF-α (kindly provided by Drs. A. Wang and L. Lin, reference 15) in 1× Denhardt's solution-10 mM DTT-300 mM NaCl-50 mM Tris-10% dextran sulfate in deionized formamide overnight at 48°C. Serial sections were also probed for HIV-1 by hybridization with pooled ^{35}S-labeled HIV-1 probes. The HIV-1 RNA probes were synthesized from cDNA cloned into pGEM4 (Promega Biotec, Madison, WI) containing both SP6 and T7 promotors and consisted of a gag/pol junction fragment (2154 basepairs), SOR fragment (604 basepairs), and envelope fragment (1055 basepairs) (Lofstrand Laboratories, Gaithersburg, MD) (14). Both the TNF-α and HIV-1 probes were synthesized using an SP6 or T7 RNA polymerase and labeled with ^{35}S-uridine triphosphate (Promega Biotec). Positively oriented (antisense) probes for TNF-α and HIV-1 were complementary to TNF-α and HIV-1 mRNA, respectively. Control (sense) probes for TNF-α and HIV-1, which were included in each corresponding hybridization, had the same sequence but reverse orientation, and therefore did not hybridize. Each hybridization also included the following control cells or tissue: elutriated human monocytes incubated for 6 h with and without LPS 10 μg/ml (positive and negative control for the TNF-α probe), HIV-infected brain from an AIDS patient who died with HIV encephalopathy (positive control for the HIV-1 probe), and normal colonic mucosa from a seronegative subject (negative control for the TNF-α and HIV-1 probes). The slides were washed sequentially in 2× SSC, 2× SSC 1 mM EDTA 5 mM DTT 0.1 Triton X at 56°C, and the same again, except with 0.1× SSC, and then treated with 40 μg/ml RNAse-10 U/ml ribonuclease T_1 (Sigma Chemical Co., St. Louis, MO) at 37°C for 40 min, washed in 2× SSC, and dehydrated in graded ethanols. Slides were prepared for autoradiography with NT-4 emul-

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1. *Abbreviations used in this paper:* CMV, cytomegalovirus; MOI, multiplicity of infection; TNF-α, tumor necrosis factor-α.

sion (Kodak, Rochester, NY), developed after 3 d and stained with hematoxylin and eosin. Localization of > 50 grains above background in a 200-μm^2 area overlying a noneosinophilic cell was defined as a positive cell.

CMV infection of monocytes. For the in vitro studies, CMV was isolated by coculturing mononuclear cells from one of the AIDS patients with CMV colitis on MRC-5 cells. CMV-containing supernatants, which had been passaged less than nine times and which did not contain HIV core p24 antigen or reverse transcriptase activity, were added at a multiplicity of infection (MOI) of either 0.1 or 1 to cultures of human monocytes (> 95% CD14 and esterase positive) purified from healthy HIV and CMV seronegative donors by counterflow centrifugal elutriation (16, 17). After culture for 24 h, CMV-exposed and mock-infected monocytes were monitored for the expression of CMV-specific early antigen, indicating infection. For detection of CMV early antigen, cytospun cells were treated for 5 min with 0.1% trypsin and then stained by the immunoperoxidase technique (18) with monoclonal antibody specific for CMV early antigen (Dako Corp., Carpinteria, CA) at a concentration of 1:10 or an irrelevant antibody (anti-HIV p24, 1:10; Dako). The CMV- and mock-infected cells were then evaluated for TNF-α gene and peptide expression as described below.

HIV-1 infection of monocytes. Monocytes were also infected with macrophage tropic HIV-$1_{Ba\text{-}L}$ in order to compare the ability of CMV and HIV-1 to induce TNF-α production. Similar to our previously described method (19, 20), monocytes (1×10^6) were cocultured in suspension for 1 h with an inoculum of HIV-$1_{Ba\text{-}L}$ containing 3.26×10^6 cpm reverse transcriptase activity (ABI, Columbia, MD) and then washed and resuspended (3×10^6 cells/1.5 ml) in RPMI 1640 (Gibco Laboratories, Grand Island, NY) without serum in culture chambers (Lab-Tek, Nunc Inc., Naperville, IL). After 2 h to allow for adherence, FCS (Sigma) was added to a final concentration of 10%. Cultures were refed with fresh media (50%) every 4 d for 28 d and the harvested media stored frozen until assayed for reverse transcriptase activity and TNF-α. Reverse transcriptase activity was assayed by the micromethod (21).

TNF-α mRNA and peptide assays. TNF-α mRNA was assayed by Northern blot analysis. Briefly, total RNA from 20×10^6 infected or control monocytes was isolated after 6 h incubation without and with LPS 10 μg/ml by acid guanidinium thiocyanate-phenol-chloroform extraction, electrophoresed (5.0 μg/lane) through a formaldehyde-containing 1% agarose gel, blotted onto nitrocellulose paper, and hybridized overnight at 37°C with ^{32}P-labeled cDNA probes for TNF-α (15) and glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) (22). Blots were washed in 5× SSC containing 0.05% sodium pyrophosphate and 0.1% SDS twice at 37°C for 45 min and twice at 55°C for 15 min and then exposed to Kodak XAR-5 film at −70°C for 48 h. The amount of TNF-α peptide released by monocytes (1×10^6/ml) cultured for 24 h after LPS 10 μg/ml stimulation in RPMI 1640 was quantitated by the L929 bioassay (23).

Results

Confirmation of CMV colitis. Cytomegalovirus colitis in four symptomatic AIDS patients was observed endoscopically (Fig. 1 *A*) and confirmed histologically by the presence of mucosal ulceration (Fig. 1 *B*), inflammatory cells (Fig. 1 *C*), and cytomegalic inclusion cells (Fig. 1 *D*), and microbiologically by isolation of CMV from the mucosal biopsies on MRC-5 cells.

Identification of TNF-α mRNA in colonic mucosa from AIDS patients with CMV colitis. The presence of cells expressing TNF-α-specific mRNA in the mucosal biopsies from the four AIDS patients with CMV colitis and the five control subjects (four asymptomatic AIDS patients and a seronegative subject with normal colonic mucosa) was evaluated by in situ hybridization using a ^{35}S-labeled cDNA probe (15). As shown in Fig. 2, *A* and *B*, mucosa from a representative AIDS patient with CMV colitis was strongly positive for TNF-α mRNA. The mRNA transcripts were present only in the lamina propria, localized to mononuclear cells with morphologic features of

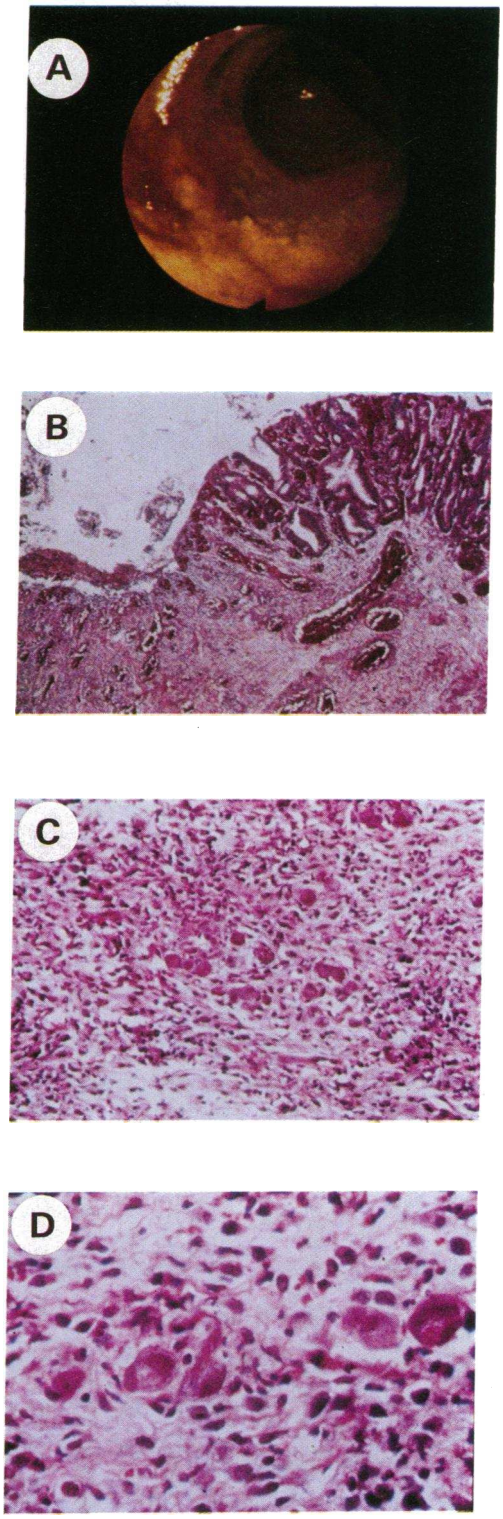

Figure 1. Endoscopic and light microscopic appearance of CMV colitis. (*A*) Direct visualization showed mucosal ulceration with active bleeding and inflammation. Light microscopy showed (*B*) ulceration and vasculitis, (*C*) infiltration by increased numbers of inflammatory cells, and (*D*) numerous cytomegalic inclusion cells. (*B*) ×30; (*C*) ×62; (*D*) ×125.

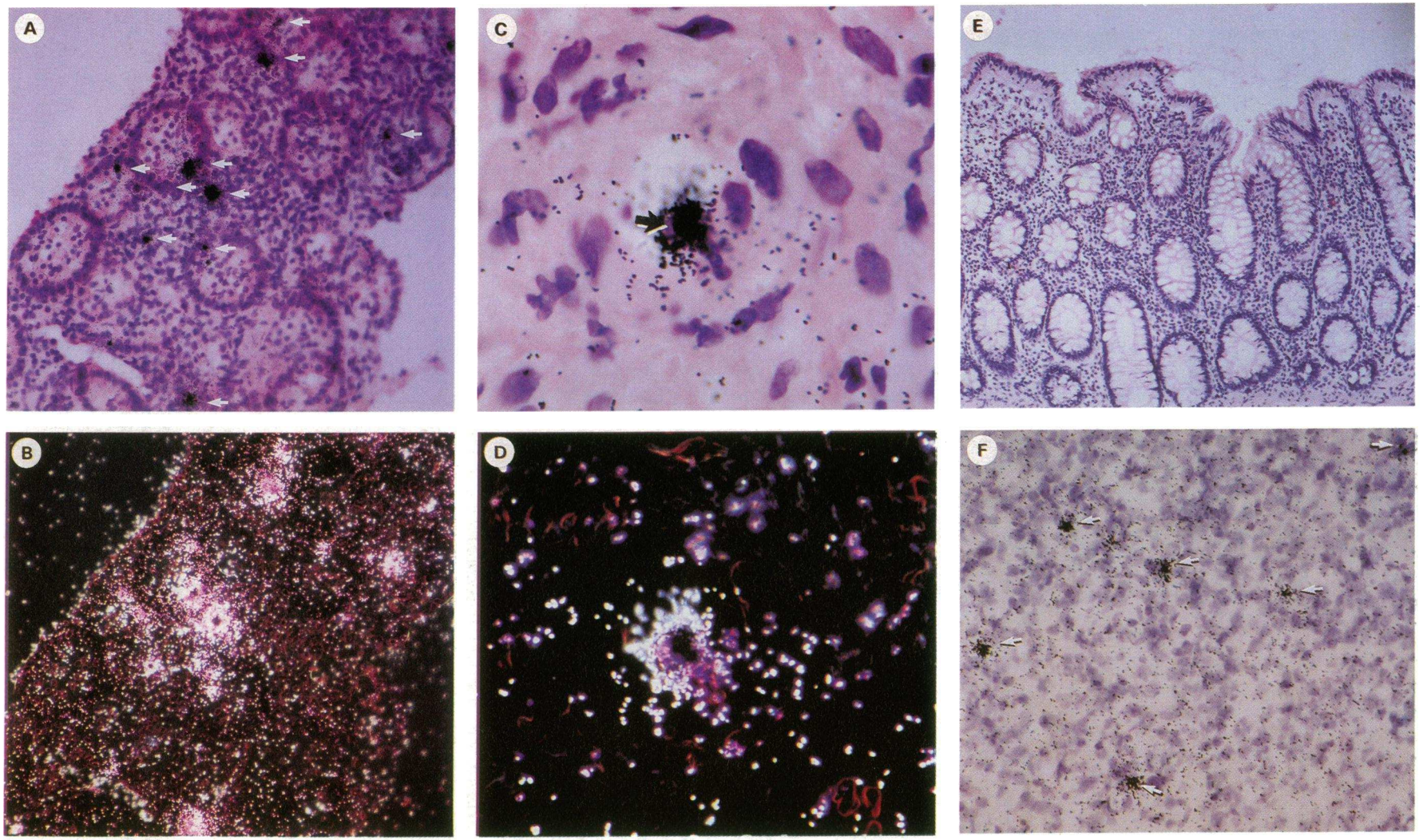

Figure 2. Localization of cells expressing TNF-α and HIV-1 mRNA in colonic tissue sections by in situ hybridization with TNF-α and HIV-1 RNA-specific (antisense) probes. (*A* and *B*) Colonic mucosa from an AIDS patient with ulcerative CMV colitis shows multiple cells expressing TNF-α-specific mRNA (*arrows*). Tissue is representative of multiple sections of colonic mucosa from 1 of 4 patients. (*C* and *D*) Colonic mucosa from another AIDS patient with CMV colitis shows a mononuclear cell that contained a cytomegalic inclusion body (*arrow*) and expressed multiple copies of TNF-α signal. (*E*) Colonic mucosa from an asymptomatic AIDS patient without CMV colitis shows no TNF-α mRNA. (*F*) Colonic mucosa from the same asymptomatic AIDS patient in (*E*) shows many mononuclear cells that expressed HIV-1-specific mRNA. Sections were hybridized with the antisense TNF-α (*A–E*) or HIV-1 (*F*) probes. Sections hybridized with control (sense) probes of the reverse (noncomplementary) orientation showed no positive cells (data not shown). (*A*, *C*, *E*, and *F*) stained with hematoxylin and eosin; bright-field illumination. (*B* and *D*) dark-field illumination. (*A*, *B*, *E*, and *F*) ×125; (*C* and *D*) ×312.

tissue macrophages, and were expressed in multiple copies by each positive cell (Fig. 2, *A–D*). Among the four patients, the number of mononuclear cells within the mucosal specimens expressing TNF-α mRNA appeared to correlate with the severity of the CMV colitis. In addition, the presence of TNF-α mRNA in cells containing cytomegalic inclusions (Fig. 2, *C* and *D*) indicates that at least some of the lamina propria monocytes/macrophages synthesizing TNF-α-specific RNA were infected with CMV. In contrast to the mucosa of AIDS patients with CMV colitis, sections of colonic mucosa from four asymptomatic AIDS patients who did not have intestinal CMV disease (Fig. 2 *E*) and a seronegative subject (data not shown) did not contain cells expressing TNF-α-specific RNA.

Although TNF-α expression by HIV-1-infected monocytes in vitro appears to be variable (24–29), peripheral blood monocytes isolated from AIDS patients have been shown to produce increased levels of TNF (30–32). Therefore, to determine whether HIV-1-infected mononuclear cells, which have been identified in AIDS patients' colonic mucosa (14), or CMV-infected cells were the primary source of the transcripts, we reprobed the patient and control tissue sections for HIV-1 mRNA. Among the four patients with CMV colitis, colon biopsies from two contained mRNA transcripts for TNF-α but not HIV-1, whereas colon biopsies from the other two contained transcripts for both TNF-α and HIV-1. Since colon biopsies from four of four patients coexpressed CMV and TNF-α, these data indicate that the presence of TNF-α mRNA in the inflamed tissue was positively associated with the CMV. In contrast, colon biopsies from the four asymptomatic AIDS patients did not contain TNF-α transcripts (Fig. 2 *E*), despite the presence of cells expressing HIV-1-specific mRNA (Fig. 2 *F*). Colon biopsies from the seronegative subject showed neither TNF-α nor HIV-1 mRNA.

CMV induction of TNF-α mRNA and peptide in vitro. Since CMV-infected cells expressed TNF-α-specific mRNA in vivo, we next sought to confirm that CMV could regulate TNF production by macrophages in vitro. Total cellular RNA was isolated from 20×10^6 monocytes that had been infected for 1 d with CMV. Control RNA was isolated from equivalent numbers of mock-infected monocytes (negative control) and monocytes treated with LPS for 6 h (positive control). TNF-α mRNA was detected by hybridization to the TNF-α cDNA probe (15). As shown in the Northern blot in Fig. 3 *A*, CMV-infected monocytes expressed message for TNF-α, whereas mock-infected monocytes did not. Levels of expression of the control GAPDH gene (22) indicate that equivalent amounts of RNA were applied to each lane. Thus, the clinical isolate of CMV induced the expression of the TNF-α gene by primary human monocytes.

Since CMV was capable of inducing synthesis of TNF-α mRNA, CMV- and mock-infected human monocytes were examined for spontaneous and LPS-triggered secretion of TNF-α. As shown in Fig. 3 *B*, neither monocytes that had been infected with CMV nor uninfected control monocytes constitutively produced detectable TNF-α bioactivity. However, after stimulation with LPS, the CMV-infected monocytes secreted levels of TNF-α that were more than fourfold greater than that of uninfected control monocytes (Fig. 3 *B*), suggesting that CMV primed the monocytes for increased sensitivity to subsequently encountered stimuli.

Monocytes exposed to CMV were also evaluated for coexpression of CMV early antigen and TNF-α mRNA. After 24 h in culture, approximately 1 per 10 to 1 per 10^2 monocytes

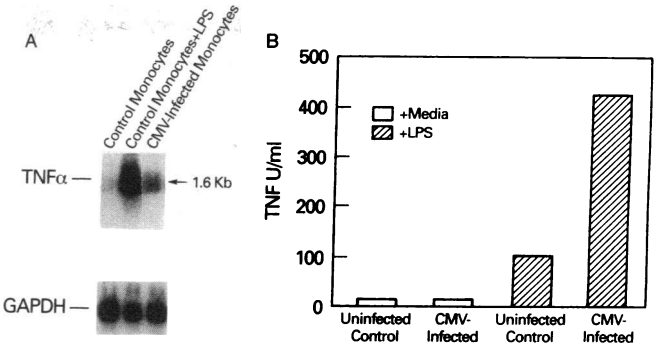

Figure 3. CMV induction of TNF-α mRNA and peptide by human monocytes. (*A*) Northern hybridization of RNA from control (mock-infected) monocytes, control monocytes stimulated with LPS 10 μg/ml, and CMV-infected (MOI 1, day 1) monocytes using ^{32}P-labeled probes for TNF-α and GAPDH. (*B*) TNF-α peptide secretion by CMV-infected (MOI 1, day 2) and uninfected monocytes without and with stimulation with LPS. Data are from a representative experiment ($n = 6$).

exposed to CMV (MOI 0.1) expressed CMV early antigen (Fig. 4 *A*), whereas the mock-infected monocytes (data not shown) and CMV-exposed monocytes stained with the irrelevant antibody (Fig. 4 *B*) were negative. This rate of (nonproductive) infection corresponds closely to rates (1% to 9%) previously reported (33, 34), although it is considerably lower than the 40% recently reported by others (35). Among monocytes in the CMV-exposed cultures, 1 per 10^2 to 1 per 10^3 monocytes expressed TNF-α transcripts (Fig. 4 *A*, *inset*), although it remains unclear whether the identical cells were CMV and TNF-α positive. Control monocytes did not express TNF-α mRNA (Fig. 4 *B*, *inset*).

Inability of HIV-1 to induce TNF-α. Although the presence of HIV-1 in the colonic mucosa of four AIDS patients with normal mucosa was not associated with the expression of TNF-α mRNA in the mucosa, we also evaluated whether HIV-1 could prime monocytes in vitro for TNF-α secretion. As shown in Fig. 5, supernatants from monocytes infected with HIV-1_{Ba-L}, taken at peak reverse transcriptase activity (Fig. 5 inset), did not contain increased amounts of TNF-α compared to uninfected control monocytes in the absence or presence of LPS. Thus, in contrast to CMV (Fig. 3 *B*), HIV-1_{Ba-L} did not prime monocytes for LPS-triggered TNF-α release, supporting previously published observations (26, 27).

Discussion

The findings reported here indicate that CMV infection of human tissue macrophages in vivo and blood monocytes in vitro is associated with the expression of TNF-α-specific mRNA. Three lines of evidence suggest that TNF-α gene expression was associated with CMV. First, TNF-α mRNA was present in inflamed colonic mucosa from AIDS patients who had CMV, even in the absence of HIV-1, in the tissue. Second, TNF-α mRNA was not present in the colon tissue of asymptomatic AIDS patients who had HIV-1 but not CMV in the tissue. This finding was corroborated by in vitro studies in which CMV, but not HIV-1, primed monocytes for increased TNF-α production. Third, CMV inclusion cells expressed TNF-α mRNA. Recently, another virus (influenza A) was shown to prime a murine cell line to produce increased amounts of TNF-α (36),

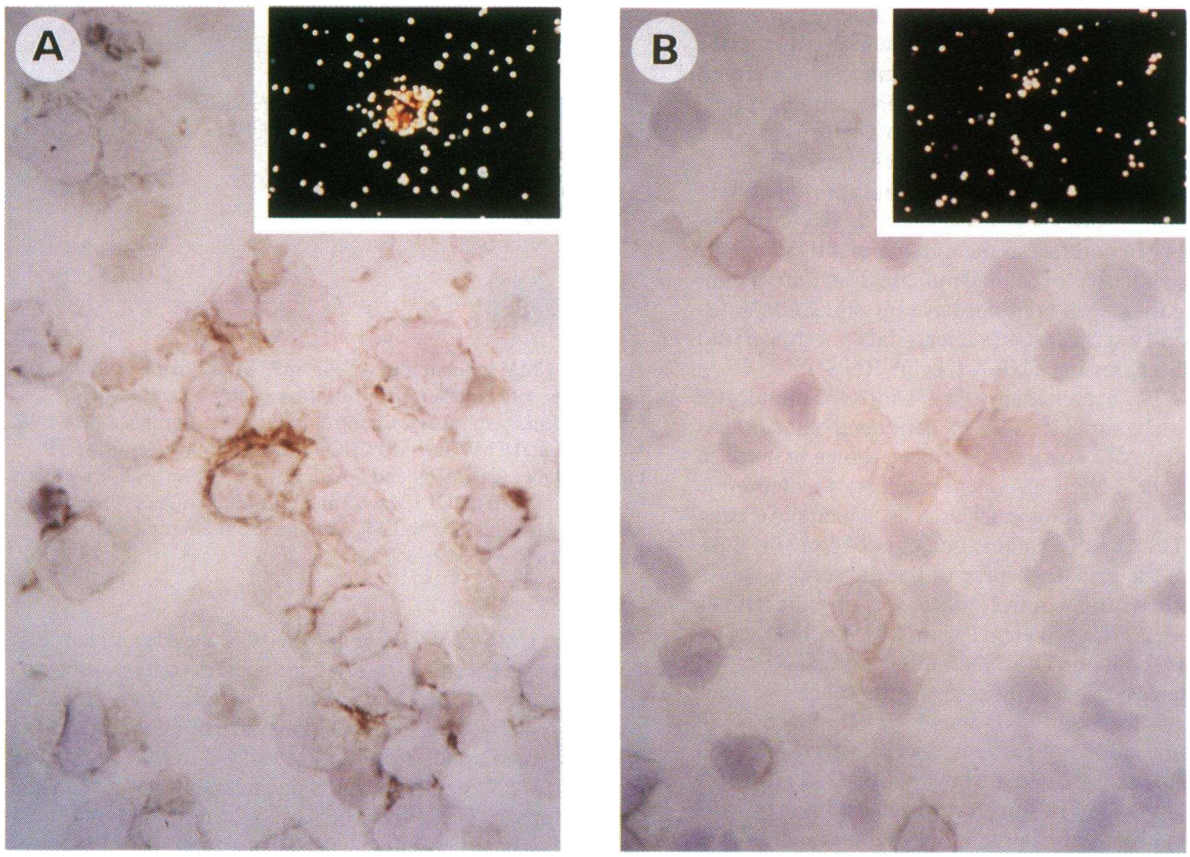

Figure 4. Detection of CMV and TNF-α in human monocytes. Primary human monocytes exposed to CMV (MOI 0.1) were cultured for two days and then stained for CMV early antigen (*A*) or isotype control antibody (anti-p24) (*B*) by the immunoperoxidase technique. The expression of TNF-α mRNA was evaluated in the CMV-exposed monocytes (*A*, *inset*) and mock-infected monocytes (*B*, *inset*) by in situ hybridization. CMV early antigen is identified by the brown reaction precipitate in the light photomicrographs (*A* and *B*; ×400); TNF-α mRNA transcripts and background are represented by the white dots in this dark field illumination (*A* and *B* insets; ×100), which correspond to silver grains in bright-field illumination.

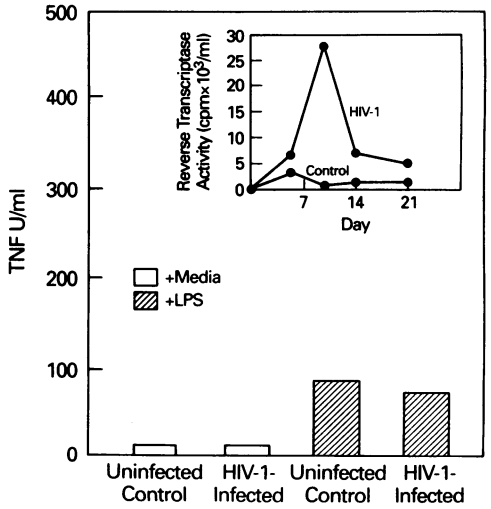

Figure 5. Inability of HIV-1 to induce TNF-α by human monocytes. Primary human monocytes exposed to HIV-1_{Be-L} and uninfected control cells were cultured for 21 d, monitored for reverse transcriptase activity, and assayed for TNF-α bioactivity released into the culture supernatant. At peak reverse transcriptase activity (*inset*), neither uninfected control nor HIV-1-infected monocytes constitutively released TNF-α. After LPS (1 μg/ml) stimulation for 24 h, uninfected control and HIV-1-infected monocytes produced equivalent amounts of TNF-α (109 vs 94 U/ml, $P > 0.05$) ($n = 2$).

and coxsackievirus B3 infection of human monocytes was shown to influence TNF-α production (37). However, this is the first report that CMV is associated with TNF-α mRNA expression in inflamed human tissue and is capable of stimulating synthesis of TNF-α mRNA and augmenting induced peptide synthesis by primary human mononuclear phagocytes. Thus, CMV infection of mucosal monocytes/macrophages is associated with the expression of TNF-α, suggesting that this cytokine plays a role in the pathogenesis of CMV colitis.

Although TNF-α may be a pivotal cytokine in CMV colitis, our observations do not exclude a role for other cytokines in mucosal inflammation. In this regard, TNF-α is not only capable of inducing its own production in an autocrine-paracrine manner, it also can stimulate production of several other cytokines, resulting in a cascading release of soluble inflammatory mediators (38). One cytokine in particular, IL-1, is an important mediator of inflammation and has been identified in certain types of colitis such as Crohn's disease colitis (39) and rabbit immune complex colitis (40). Interleukin 6, which has activities that overlap those of IL-1 and TNF-α (41) and may be inducible at mucosal surfaces (42), and platelet-activating factor, which induces bowel necrosis in animals (43, 44), may also contribute to CMV-associated mucosal inflammation.

Supporting the notion that TNF-α mediates mucosal inflammation are observations in rodents that recombinant TNF-α can directly induce bowel ischemia, hemorrhage, and

necrosis accompanied by bloody diarrhea (45) and promote the intestinal inflammatory lesion in graft-versus-host disease (46, 47). The presence of receptors for TNF-α in both the small and large intestine (48) could facilitate these inflammatory reactions. That in vitro expression of TNF-α peptide by CMV-infected monocytes required secondary stimulation is particularly relevant to the gastrointestinal tract which is constantly being exposed to potential macrophage stimulants, such as bacterial LPS. By parallel mechanisms, TNF-α could contribute to the pneumonitis and other inflammatory reactions commonly associated with CMV-induced organ disease in patients with AIDS. In this regard, the source for CMV-infected tissue macrophages is likely peripheral blood mononuclear cells, the major reservoir for CMV in healthy persons (1–3). Finally, the findings reported here extend a previous observation that heat-inactivated CMV antigen stimulated monocytes to release unidentified soluble products which upregulated HIV-1 expression from chronically infected cell lines (49). Since TNF-α is capable of upregulating HIV-1 expression (10, 11), the ability of infectious CMV to stimulate TNF-α gene and peptide expression from human monocytes offers a potential mechanism by which CMV could act as a co-factor for HIV-1 expression without requiring that both CMV and HIV-1 infect the same cell.

Acknowledgments

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