Infection with cytomegalovirus in patients with inflammatory bowel disease: prevalence, clinical significance and outcome

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Despite frequent use of immunosuppressive drugs in patients with inflammatory bowel disease (IBD) and reports of cytomegalovirus (CMV) infection following post-transplant immunosuppression, data on the frequency and clinical significance of CMV in patients with IBD are scant. Sixty-three patients with IBD (61 ulcerative colitis and two Crohn’s disease) were evaluated for CMV using serology (IgM antibody, /C236 capture ELISA), PCR for CMV DNA in colonic biopsy and histological assessment of haematoxylin and eosin-stained colonic biopsy. Positive result in any test was considered as CMV infection. Various parameters associated with CMV infection were analysed using univariate and multivariate analysis. Ten of 63 (15.8 %) patients (age 36.0 ± 11.2 years, 31 female) were infected with CMV (DNA alone in four, IgM antibody alone in two and both in four, inclusion body in one). Patients with CMV infection were more often female (8/10 vs 23/53, P = 0.05), had pancolitis (10/10 vs 33/53, P = 0.05), histological activity (9/10 vs 17/53, P = 0.005) and used azathioprine (5/10 vs 7/53, P = 0.04; Fisher exact test for all). On multivariate analysis, female gender, pancolitis and histological activity were the independent factors associated with infection. Patients with CMV infection more often required surgical treatment for IBD (4/10 vs 4/53, P = 0.01) and had fatal outcome (3/10 vs 0/53, P = 0.003). CMV infection in patients with IBD may be common and is associated with poor outcome. PCR of rectal biopsy was the most sensitive method of detection followed by IgM antibody for diagnosis.

INTRODUCTION

Infection with cytomegalovirus (CMV) is an important cause of morbidity and mortality after solid organ (kidney and liver) transplantation, as these patients receive multiple immunosuppressive drugs (Patel & Paya, 1997; Wiesner et al., 1993). Patients with inflammatory bowel disease (IBD), particularly those with severe, corticosteroid-refractory and -dependent states are frequently treated with immunosuppressive agents including corticosteroids, cyclosporine, azathioprine and methotrexate, either alone or in combination (Kho et al., 2001). Therefore, patients with IBD [ulcerative colitis (UC) and Crohn’s disease (CD)] are expected to be at an increased risk of infection with CMV. However, data on CMV infection in patients with IBD is still scant and most studies used only histology and/or serology for diagnosis of CMV infection (Powell et al., 1961; Cooper et al., 1977; Berk et al., 1985; Eyre-Brook & Dundas, 1986; Vega et al., 1999; Cottone et al., 2001). Although histology is quite specific, it is of low sensitivity (Beaugerie et al., 1997). PCR has emerged as the most sensitive method for diagnosis of viral infection including that with CMV (Storch et al., 1994). However, only a few studies used PCR for diagnosis of CMV infection in IBD. With blood and buffy coat preparation of leukocytes as specimens, PCR failed to detect CMV in blood of patients with IBD in some of these studies (Adani et al., 2001). Demonstration of CMV DNA in colonic tissue may provide more direct evidence of infection with CMV in patients with IBD, as infections with CMV tend to localize to the colon, although disseminated disease has also been reported (D’Haens et al., 1998).

UC is common all over the world, including India, and is usually more frequent than CD (Sood et al., 2003). Presentation of acute attack of UC and of CMV colitis have been reported to be similar (Kaufman et al., 1999; Ng et al., 1999). Since immunosuppression can lead to flare-up of CMV infection, outcome of patients with an acute attack of UC and CMV infection is likely to be worse, if treatment with immunosuppressive drugs is continued without treating the CMV infection. A few anecdotal cases and uncontrolled

Abbreviations: CD, Crohn’s disease; CMV, cytomegalovirus; H & E, haematoxylin and eosin; IBD, inflammatory bowel disease; UC, ulcerative colitis.
series with severe UC not responding to immunosuppressive drugs did respond to treatment for CMV infection (Pflau et al., 2001; Malhi et al., 2003). Accordingly, we prospectively studied patients with IBD for (a) frequency of infection with CMV, (b) relationship of infection with CMV with severity and outcome of medical treatment of IBD and (c) comparison of various tests for detection of this infection.

METHODS

Patients. Sixty-three patients with IBD (both UC and CD) attending the Luminal Gastroenterology Clinic or admitted to a tertiary referral centre in northern India were included in this study. Diagnosis of UC and CD was based on clinical, endoscopic, radiological and histological parameters (Pera et al., 1987). Informed consent was obtained from each patient before inclusion in the study. Institutional Ethics Committee approved the study protocol.

Assessment of severity of disease. Severity of UC and CD at the time of collection of specimens for CMV was assessed using Truelove and Witts’ criteria and Harvey–Bradshaw activity index, respectively (Truelove & Witts, 1955; Harvey & Bradshaw, 1980).

Treatment received before inclusion into the study. Data on drugs used in treatment for IBD prior to collection of the specimens for study of CMV infection was noted, with particular attention to treatment with systemic and local steroid, azathioprine, methotrexate and cyclosporine. The dose of these drugs and duration of administration were recorded.

Follow-up. Patients were followed-up in the Luminal Gastroenterology Clinic of the Department of Gastroenterology. They were treated with corticosteroid during acute attacks and 5-aminosalicylic acid for maintenance of remission.

Colonoscopic assessment for the extent and activity of IBD. A full-length colonoscopy was performed using a video colonoscope (Pentax) in all patients either at the time of collection of specimens or during follow-up, as patients with severe attack of IBD are at increased risk of perforation during full-length colonoscopy (Marion & Present, 1997). Colonoscopic inflammation was graded as described previously by Baron et al. (1964); briefly, grade 0: normal mucosa; grade 1: loss of vascular pattern; grade 2: granular, non-fimbriated mucosa; grade 3: friability on rubbing; grade 4: spontaneous bleeding, ulceration. Pancolitis was diagnosed at colonoscopy if the disease involved colon proximal to hepatic flexure; the disease limited to splenic flexure was considered as left-sided colitis (Pera et al., 1987).

Collection of specimens. Multiple biopsies were obtained during colonoscopy for histological examination of inflammatory activity and CMV inclusion body in buffered neutral formalin, and for extraction of DNA for PCR in normal saline. Colonoscopic biopsies collected in normal saline were stored at 80 °C until further processing for CMV DNA. Five millilitres venous blood was obtained from each patient under aseptic precautions for serological studies.

Histopathology. Colonic biopsies, fixed in buffered neutral formalin, were paraffinized, sectioned and stained with haematoxylin and eosin (H & E). These sections were evaluated under a microscope for characteristics cytomegalic cells and ‘owl’s-eye’ nuclear inclusion bodies. Histologically, activity of IBD was classified according to a standard system described previously by Truelove & Richards (1956). Briefly, absence of any significant inflammation was considered as remission and heavy inflammatory infiltrate with epithelial ulceration as active disease; some degree of inflammatory infiltrate without epithelial ulceration was considered as moderately active disease.

Serology. Anti-CMV IgM antibodies were tested in all sera by capture ELISA using a commercially available kit (Organon Teknika) using the positive and negative controls provided with the kit.

Detection of CMV DNA by PCR in colonic tissues

Extraction of DNA from tissues. Frozen biopsy specimens were thawed, immersed in liquid nitrogen and after evaporation of liquid nitrogen homogenized using pestle and mortar. Ten volumes of extraction buffer (containing 10 mM Tris/HCl at pH 8, 0.1 M EDTA at pH 8, 0.5 % SDS) and protease K (200 µg ml−1) were added to the homogenates. The resultant mixture was placed in a water bath at 50 °C for 3 h and then heated at 95 °C for 10 min to inactivate protease K. Supernatant obtained after centrifugation and protease K digestion was subjected to DNA extraction, once with equal volume of phenol/chloroform and once with phenol/chloroform/isoamyl alcohol (25:24:1). DNA was precipitated with double volume of cold absolute ethanol and sodium acetate (3 mM), for 1 h at −70 °C and centrifuged at 12 000 r.p.m. for 20 min at 4 °C. The DNA pellet was washed with 70% ethanol, dried and resuspended in 40 µl sterile distilled water. The quality and quantity of DNA were determined spectrophotometrically at 260 and 280 nm.

Amplification of DNA by PCR. PCR was performed on colonic biopsies by standard technique using previously described oligonucleotides from the HindIII–X fragment region. Sequence of the forward and reverse primers used in PCR has been previously reported to be highly sensitive (94% in patients with symptomatic CMV infection; Mendez et al., 1998): 5′-GGATCCGCGATGCACTACGTTG-3′, 5′-GAATTCA TGGATAACCTGCGGCGA-3′. PCR was performed in a reaction mixture containing 5 µl target DNA, 100 pM each oligonucleotide primer, 1.25 U Taq polymerase, 200 mM each dATP, dTTP, dGTP, dCTP, 5 µl 10× reaction buffer (Bangalore Genei) and HPLC-grade distilled water to a total volume of 50 µl. PCR tubes were subjected to 35 cycles of amplification (94 °C for 1 min, 55 °C for 2 min and 72 °C for 3 min with final extension at 72 °C for 10 min) in a DNA thermal cycler (MJ Research). Positive and negative controls were run for each PCR batch. Amplified PCR products were electrophoresed on 2% agarose gels, stained with ethidium bromide and visualized with UV light. Presence of a 406 base pair amplicon was considered as CMV DNA.

Criteria for diagnosis of CMV infection. Positive result in one or more of the three tests (IgM antibody, CMV DNA and inclusion body in H & E-stained sections) was considered as evidence of CMV infection.

Evaluation for associated human immunodeficiency virus (HIV) infection. Patients with evidence of CMV infection were evaluated for associated HIV infection using anti-HIV 1 and 2 antibody enzyme immunoassays (HIV chex, Qualigenes Diagnostics and Comb AIDS, Span Diagnostics, respectively).

Statistical analysis. Categorical and continuous data were compared using two-tailed Fisher exact test and Mann–Whitney U test, respectively. Parameters found to be associated with infection with CMV were subsequently entered into a multivariate model (discriminant analysis) to evaluate independent factors associated with infection with CMV. P values below 0.05 were considered significant. Sensitivity, positive and negative predictive values and accuracy of each test were calculated considering positive result in any of the three tests for CMV as evidence for infection.
RESULTS AND DISCUSSION

Prevalence of active CMV infection

The demographic and clinical parameters of patients with or without CMV infection are summarized in Table 1. Most patients were young adults (age 36.0 ± 11.2 years, 31 female). Of 63 patients, ten (15.8%) had evidence of CMV infection [CMV DNA alone in four (Fig. 1), serum anti-CMV IgM antibodies alone in two and both in four patients]. Only one patient had characteristic CMV inclusion bodies in H & E stained tissue section from colonic biopsy (Fig. 2). This patient had IgM anti-CMV antibody in serum but PCR for CMV in endoscopic biopsy was negative. None of the ten patients infected with CMV had a positive anti-HIV antibody test.

Table 2 shows the utility of various tests in detection of CMV in patients with IBD. Sensitivity of PCR, IgM antibody in serum and histological examination for CMV inclusion body on H & E-stained colonic tissue was 80, 60 and 10%, respectively.

Histological examination of colonic biopsies had the lowest sensitivity, probably because the characteristic inclusion bodies are not readily visible in routinely performed H & E stain (Beaugerie et al., 1997) and because CMV-infected cells are not always cytomegalic. As it was suggested that CMV inclusion bodies are more often found in the right colon than in the left (Hinnant et al., 1986), multiple biopsies were taken

Table 1. Demographic and clinical parameters of patients (n = 63) with IBD with or without CMV infection

Continuous data are expressed as median and range (in brackets). The categorical and continuous variables are compared using two-tailed Fisher exact test and Mann–Whitney U test, respectively. Numbers in parentheses indicate percentage. NS, Not significant.

<table>
<thead>
<tr>
<th>Variable</th>
<th>CMV +ve (n = 10)</th>
<th>CMV -ve (n = 53)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>39 [18–58]</td>
<td>36 [16–67]</td>
<td>NS</td>
</tr>
<tr>
<td>Gender (female)</td>
<td>8 (80)</td>
<td>23 (43-4)</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>Duration of disease (months)</td>
<td>30 [6–120]</td>
<td>21 [0.5–240]</td>
<td>NS</td>
</tr>
<tr>
<td>Crohn’s disease</td>
<td>1 (10)</td>
<td>1 (2)</td>
<td>NS</td>
</tr>
<tr>
<td>Fulminant/severe disease</td>
<td>8 (80)</td>
<td>28 (52-8)</td>
<td>NS</td>
</tr>
<tr>
<td>Pancolitis at colonoscopy</td>
<td>10 (100)</td>
<td>33 (62-3)</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>Active disease at colonoscopy</td>
<td>10 (100)</td>
<td>39 (73-6)</td>
<td>NS</td>
</tr>
<tr>
<td>Active disease at histology</td>
<td>9 (90)</td>
<td>17 (32-1)</td>
<td>0.005</td>
</tr>
<tr>
<td>Recent corticosteroid</td>
<td>10 (100)</td>
<td>44 (83)</td>
<td>NS</td>
</tr>
<tr>
<td>Azathioprine*</td>
<td>4 (40)</td>
<td>6 (11)</td>
<td>0.04</td>
</tr>
<tr>
<td>Cyclosporine*</td>
<td>1 (10)</td>
<td>1 (1-8)†</td>
<td>NS</td>
</tr>
<tr>
<td>Outcome</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Surgery‡</td>
<td>4 (40)</td>
<td>4 (7-5)</td>
<td>0.01</td>
</tr>
<tr>
<td>Death§</td>
<td>3 (30)</td>
<td>0 (0)</td>
<td>0.003</td>
</tr>
</tbody>
</table>

*All these patients received corticosteroid as well.
†This patient received cyclophosphamide as well as cyclosporine for steroid refractory colitis with peripheral vasculitis [described previously; Kumar et al. (2004)].
‡One patient each in CMV +ve and —ve groups had peritoneal toilet for management of perforation; all others had colectomy.
§Two patients died of post-operative complications and one of azathioprine-induced pancytopenia and septic shock.
PCR of colonic biopsy was the most sensitive method to diagnose CMV infection. As most adult Indians are IgG anti-CMV seropositive, we did not evaluate IgG antibody. Considering the high prevalence of anti-CMV IgG in adult Indians, it is probable that most cases of active CMV infection detected in this study are CMV reactivation and not primary CMV infection. It is possible that if in situ hybridization (Paya et al., 1990), immunohistochemistry (Paya et al., 1990) and/or immunocytochemical staining of CMV pp65 early antigen in lymphocytes (Yakushiji et al., 2002) were used, a larger number of patients with active CMV infection might have been diagnosed. Therefore, the possibility of underestimation of CMV infection in this study cannot be entirely excluded. However, this is unlikely, because the frequency of CMV infection was either similar or somewhat higher than in most previous studies, which reported widely varying prevalences of active CMV infection (0.53–36 %) in patients with IBD (Papadakis et al., 2001; Vega et al., 1999; Cottone et al., 2001). This widely varying frequency of CMV infection might be related to the different patient populations studied, treatment used prior to inclusion into the study or types of specimens and different diagnostic techniques used. In fact, the study that reported 36 % frequency of CMV infection, evaluated 19 of 62 severe corticosteroid-refractory patients and not all 62 patients with IBD (Cottone et al., 2001). However, it is likely that the frequency of CMV infection in patients with IBD has been underestimated in other reports, as these patients are often treated with multiple immunosuppressive drugs, which are known to be quite frequently associated with CMV infection after solid organ transplantation (Kho et al., 2001; Sia & Patel, 2000).

**Factors associated with CMV infection**

Table 1 summarizes association of various clinical variables with CMV infection. Patients with evidence of CMV infection were more often female, had pancolonic disease at colonoscopy, active inflammation at histology, had been more often using azathioprine in addition to corticosteroid than those without CMV infection. Six of 53 (11 %) and four of ten (40 %) patients in CMV-negative and -positive groups were on azathioprine treatment, respectively (P < 0.05, Fisher exact test). In addition, one patient in each group was on cyclosporine. The patient on cyclosporine in the CMV-negative group was also treated with cyclophosphamide for peripheral vasculitis. Patients with CMV infection more often required surgical treatment and died due to IBD than those without CMV infection. Indications for surgery included colonic perforation in one patient each in CMV-infected and non-infected group, and non-response to medical treatment in all others. Three of ten patients in CMV-infected group died; one of them succumbed to pancytopenia and fulminant septicemia following treatment with azathioprine for corticosteroid non-responsive CD. The other two patients died in the post-operative period, one of whom had colonic perforation and both had intra-abdominal sepsis and septicemia.

On multivariate analysis, female gender, pancolonic disease upon colonoscopy and histologically active disease were the independent factors associated with CMV infection. Eight of 31 (25.8 %) female patients with IBD had infection with CMV; in contrast, two of 32 (6.2 %) males had CMV infection (P = 0.04, two-tailed Fisher exact test). Female preponderance in the CMV-infected group, as observed in the current study, has already been described (Waya et al., 2003).

**Clinical significance of active CMV infection in IBD patients**

In the current study, CMV infection was an independent parameter associated with need for surgical treatment and/or...
mortality in these patients upon multivariate analysis. In a previous study of nine patients with CMV infection and IBD, two required surgery (Vega et al., 1999), in another study, 11 of 22 patients with IBD had to undergo colectomy despite receiving immunosuppressive treatment with steroids (Kaufman et al., 1999). However, these reports were retrospectively collected case series and are therefore, prone to limitations.

To our knowledge, this is the first prospective study attempting to evaluate clinical significance of CMV infection in patients with IBD using multivariate analysis. Our results showed that pancolitis and histologically active disease were independently associated with CMV infection. Treatment with azathioprine in addition to corticosteroids was another factor significantly associated with CMV infection. This is not surprising as CMV colitis is a well known complication of immunosuppression such as AIDS, transplantation, malignancies and during treatment with chemotherapy and corticosteroids (Goodgame, 1993; Sia & Patel, 2000).

Interestingly, the outcome of medical treatment for IBD in patients with CMV infection was worse than those without CMV. Patients with CMV infection more often required colectomy and died. One of the three patients who died in the CMV-infected group in the current study developed colonic perforation; the other two died in the post-operative period with sepsicaemia, which might have started before surgery due to unrecognized colonic perforation and intra-abdominal sepsis. As CMV colitis is known to cause toxic megacolon and colonic perforation (Cooper et al., 1977; Eyre-Brook & Dundas, 1986; Toogood et al., 1996), and CMV colitis is expected to be more severe with continuing immunosuppressive treatment, complications like colonic perforation in IBD patients can be due to CMV colitis. Therefore, we believe that patients with steroid-refractory or -dependent IBD, particularly females with active pancolitis, should be screened for CMV infection before increasing the dose and number of immunosuppressive drugs, as already suggested by Bloomfeld (2001). However, there is no consensus about how to manage IBD patients if active CMV infection is diagnosed. Considering the results of the present study and knowledge about the risk of high-dose immunosuppression in the presence of active CMV infection, one may tend to believe that a therapeutic strategy to combat CMV is warranted. With the currently available data, a reduction of the dose of corticosteroids and other immunosuppressive drugs is unlikely to be acceptable in the presence of active severe IBD. Therefore, treatment with antiviral agents such as gancyclovir is the only potential option. However, only a few case reports and uncontrolled series support this currently (Cottone et al., 2001; Pfau et al., 2001; Malhi et al., 2003; Papadakis et al., 2001). In fact, guidelines for treatment of CMV infection of the bowel are only specified for AIDS patients (Whitley et al., 1998). Therefore, further studies on this issue are urgently necessary.

Although a few reports suggested an aetiological role of CMV in IBD (Diepersloot et al., 1990; Lortholary et al., 1993), we believe that reactivation of CMV in immunosuppressed patients with IBD has clinical implications due to development of secondary CMV colitis rather than its role in aetiology of IBD. TNF-α and IFN-γ, which are elevated in patients with IBD, have been suggested to cause reactivation of latent CMV infection (Soderberg-Naucler et al., 1997); this is known to cause liberation of pro-inflammatory cytokines such as IL6, which might cause exacerbation of IBD (Rahbar et al., 2003). Corticosteroid and other immunosuppressives, though suppressing immune-mediated intestinal injury, further reactivate latent CMV infection. Despite a small sample size, our data do suggest that reactivation of CMV infection in patients with IBD is associated with risk of complications, need for surgical treatment for IBD, and mortality; these need to be substantiated in more studies with larger numbers of patients.

In conclusion, CMV infection in patients with IBD may be common and is often underestimated. This has definite clinical significance and therefore should not be ignored. Further studies including randomized controlled trials on anti-CMV treatment in such patients are needed.

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