

Prospective Study

Human cytomegalovirus and Epstein-Barr virus infection in inflammatory bowel disease: Need for mucosal viral load measurement

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Abstract

AIM: To evaluate the best diagnostic technique and risk factors of the human Cytomegalovirus (HCMV) and Epstein-Barr virus (EBV) infection in inflammatory bowel disease (IBD).

METHODS: A cohort of 40 IBD patients (17 refractory) and 40 controls underwent peripheral blood and endoscopic colonic mucosal sample harvest. Viral infection was assessed by quantitative real-time polymerase chain reaction and immunohistochemistry, and correlations with clinical and endoscopic indexes of activity, and risk factors were investigated.

RESULTS: All refractory patients carried detectable levels of HCMV and/or EBV mucosal load as compared

to 13/23 (56.5%) non-refractory and 13/40 (32.5%) controls. The median DNA value was significantly higher in refractory (HCMV 286 and EBV 5.440 copies/ 10^5 cells) than in non-refractory (HCMV 0 and EBV 6 copies/ 10^5 cells; $P < 0.05$ and < 0.001) IBD patients and controls (HCMV and EBV 0 copies/ 10^5 cells; $P < 0.001$ for both). Refractory patients showed DNA peak values $\geq 10^3$ copies/ 10^5 cells in diseased mucosa in comparison to non-diseased mucosa ($P < 0.0121$ for HCMV and < 0.0004 for EBV), while non-refractory patients and controls invariably displayed levels below this threshold, thus allowing us to differentiate viral colitis from mucosal infection. Moreover, the mucosal load positively correlated with the values found in the peripheral blood, whilst no correlation with the number of positive cells at immunohistochemistry was found. Steroid use was identified as a significant risk factor for both HCMV ($P = 0.018$) and EBV ($P = 0.002$) colitis. Finally, a course of specific antiviral therapy with ganciclovir was successful in all refractory patients with HCMV colitis, whilst refractory patients with EBV colitis did not show any improvement despite steroid tapering and discontinuation of the other medications.

CONCLUSION: Viral colitis appeared to contribute to mucosal lesions in refractory IBD, and its correct diagnosis and management require quantitative real-time polymerase chain reaction assay of mucosal specimens.

Key words: Inflammatory bowel disease; Quantitative real-time polymerase chain reaction; Steroid therapy; Refractory; Viral infection

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Core tip: This study investigated the presence of human Cytomegalovirus and Epstein-Barr virus (EBV) infection in patients with refractory and non-refractory inflammatory bowel disease (IBD). We identified quantitative real-time polymerase chain reaction assay of mucosal specimens as the best diagnostic technique. This allowed us to distinguish between viral colitis and infection through the identification of a cutoff value. All refractory IBD patients carried the highest mucosal viral loads, which correlated with the severity of mucosal damage and endoscopic activity. EBV infection was the most prevalent. Finally, steroid therapy was identified as a significant risk factor for viral colitis.

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INTRODUCTION

Growing evidence highlights the role of early treatment of inflammatory bowel disease (IBD) by means of a more aggressive therapy, in order to achieve mucosal healing and prevent disease progression^[1]. As a consequence, a sizeable number of patients are on immunosuppressive and/or biological drugs which, in turn, lead to an increased risk of opportunistic infections^[2,3]. Among these, the widespread human cytomegalovirus (HCMV) and Epstein-Barr virus (EBV) are capable of establishing latency in target cells and reactivating in cases of reduced host immunity^[4], giving rise to both systemic and end-organ disease, which can also be localized localized to the gastrointestinal tract^[5,6]. In addition, an increased risk of lymphoma has been recently found in IBD patients under immunosuppressive and/or immunomodulator therapies, especially among young males under thiopurines, where a role for primary EBV infection has been proposed^[7], as in the case of the post-transplant lymphoproliferative disease^[8]. A further condition now possibly associated with both HCMV and EBV infections is hemophagocytic lymphohistiocytosis^[9].

To date, the information available on the frequency, role and risk factors of HCMV and EBV in IBD exacerbation and their diagnostic and therapeutic approaches is conflicting^[10,11]. The causes for this discrepancy lie in the differences amongst the patients enrolled, the diagnostic methods applied, and the retrospective design of the majority of studies. Herein, we prospectively evaluated the presence of HCMV and EBV infections in a cohort of IBD patients in comparison to control subjects, through both immunohistochemistry and quantitative real-time polymerase chain reaction (PCR), in order to investigate the contribution of these opportunistic viral infections to disease activity and the risk factors for their reactivation.

MATERIALS AND METHODS

Study population

From September 2011 to February 2013, patients suffering from Crohn's disease (CD) and ulcerative colitis (UC), both responders and non-responders to conventional therapies, and diagnosed on the basis of widely accepted criteria^[12,13], were prospectively enrolled. For the non-responder group, steroid-refractoriness was defined as the persistence of active disease despite prednisolone, or equivalent, of up to 0.75 mg/kg per day over a period of four weeks^[12,13]; primary and secondary non-response to anti-tumor necrosis factor- α agents, *i.e.*, infliximab and adalimumab, was considered as the lack of clinical improvement with induction therapy or re-

currence of disease activity during maintenance therapy despite an appropriate interval adjustment and dose escalation with exclusion of concomitant conditions, respectively^[14]; and resistance to immunosuppressive therapy, *i.e.*, Azathioprine, after the dosage adjustment was carried out on the basis of the erythrocyte levels of the active metabolite, 6-thioguanine nucleotide^[15]. Finally, for those patients under combination therapies, refractoriness was defined as the persistence of active disease despite a treatment duration of at least four weeks. All patients underwent lower endoscopy as part of their diagnostic workup for disease relapse or follow-up. Patient assessment was performed according to the Montreal classification^[16], and also included: clinical examination, body mass index calculation, evaluation of smoking habits, routine laboratory tests, and assessment of both clinical (CD activity index: CDAI^[17] and colitis activity index: CAI^[18]) and endoscopic (SES-CD^[19] and Baron^[20]) indexes of activity. As a control group, sex- and age-matched subjects undergoing lower endoscopy for irritable bowel syndrome or screening for polyps, who were not taking any drugs, were recruited. The presence of concomitant autoimmune diseases, primary immunodeficiencies, cancer or organ failure was considered exclusion criteria.

Quantitation of HCMV and EBV DNA

For each patient and control, the HCMV and EBV load was assessed in terms of DNA copies on both freshly collected peripheral blood samples and endoscopic specimens harvested from all colonic segments (right, transverse, descending, and sigma-rectum), by quantitative real-time PCR technique as previously reported^[21]. Specifically, in the IBD group, biopsies were taken from both inflamed and healthy mucosa as assessed during the endoscopic examination, that is from the edge of the ulcers and the nearby damaged zones for the former and at least 20 cm away from the affected areas for the latter. Viral DNA extraction was performed by using the NucliSENS[®] easyMAG[®] kit (BioMérieux; Lyon, France). Results were expressed as viral DNA copies/ml blood and copies/10⁵ cells. Normalization of HCMV and EBV DNA load in tissue samples was obtained by quantitative determination of β_2 -microglobulin gene^[22]. The lower detection limit was 10 DNA input target DNA copies^[21,22].

Immunohistochemistry

Mucosal specimens harvested in parallel from the same areas as those for the PCR assay, were fixed in 10% neutral buffered formalin and paraffine-embedded. Sections (5 μ m) were transferred to pre-treated glass slides (DAKO, Denmark) and stored at 37 °C overnight. The hematoxylin-eosin staining was performed following standard protocol, while the specific immunostaining for HCMV and EBV was carried out on serial sections after microwave demasking treatment. The slides were then washed

and incubated for two hours with the following mouse monoclonal antibodies: anti-HCMV (clones CCH2 and DDG9 that recognize HCMV immediate-early and early antigens, respectively, at 1:300 dilution; DAKO), and anti-LMP1 (clone CS1-4 reactive to EBV latent membrane protein-1, at 1:100 dilution; DAKO). Finally, a universal biotinylated secondary antibody (DAKO) was applied, followed by the usual reactions to allow color development (liquid DAB + Substrate Chromogen System; DAKO) and counterstaining with Harris' hematoxylin. Appropriate positive controls and non-immune protein-negative controls were used, and positive cells were evaluated by a pathologist blinded to patient diagnosis and clinical disease status.

Statistical analysis

Baseline demographic and disease features are presented by using descriptive statistics. As such, continuous variables were described as median, while categorical variables were expressed as counts and percentages, all with range. The univariate analysis was carried out to compare data between groups by applying the following tests: Fisher's exact test or Wilcoxon matched pairs signed-ranks, and Mann-Whitney or Kruskal-Wallis, for categorical and continuous variables, respectively, as appropriate. The multiple regression analysis was performed to assess the association between possible risk factors and the occurrence of viral infections. The individual demographic and clinical variables (including gender, age, body mass index, smoking habits, duration of both therapies and disease, concomitant immunosuppressive therapy, clinical and endoscopic indexes of activity) were considered as risk factors. The Spearman rank correlation test was applied to measure the association between continuous variables, and the calculation of the OR at 95%CI was assessed. GraphPad InStat 3.0 was used for computation. A 2-sided *P*-value ≤ 0.05 was considered statistically significant.

Ethical considerations

All samples were collected for diagnostic purposes and only residual aliquots of both peripheral blood and mucosal specimens from control subjects were used, after the subjects had signed the informed consent, and in accordance with the recommendations of the local Bio-Ethics Committee.

RESULTS

Study population

A total of 17 refractory and 23 non-refractory IBD patients, and 40 control subjects whose clinical features are presented in Table 1 were consecutively recruited at the Department of Internal Medicine, IRCCS Policlinico San Matteo Foundation (Pavia, Italy). Specifically, all refractory patients were admitted to the hospital for severe active disease, while in the

Table 1 Demographic and clinical features of inflammatory bowel disease patients

	Responders	Refractory	Controls	P value
Number of patients	23	17	40	
Male/female	14/9	9/8	24/16	
Body mass index ¹	22.8 (19.9-26.4)	20.3 (16.4-22.5)	23.4 (18.9-27.3)	
Age (yr) ¹	45 (16-68)	50 (16-61)	46 (17-68)	NS
CDAI score ¹	145 (124-205)	383 (265-595)	NA	0.002
Number of patients	12	4		
Location	L1 = 2 L2 = 3 L3 = 7 L4 = 0	L1 = 0 L2 = 0 L3 = 4 L4 = 0		NS
CAI score ¹	6 (3.5-9.5)	10 (8.25-11)	NA	0.040
Number of patients	11	13		
Location	E1 = 2 E2 = 3 E3 = 6	E1 = 0 E2 = 5 E3 = 8		NS
Endoscopic activity indexes			NA	
SES-CD	6.2	10.6		0.010
Baron	1.4	2.6		0.050
Illness duration (yr) ¹	6.5 (1.75-11)	5 (1-7)	NA	NS
Systemic steroids	4	14	NA	0.001
Topical steroids	5	8	NA	NS
Biological agents	5	8	NA	NS
Azathioprine	7	5	NA	NS
Biological + steroid	0	6	NA	0.0032
Biological + azathioprine	1	4	NA	NS
Azathioprine + steroid	1	0	NA	NS

¹The values are given as median (range). L1: Terminal ileum; L2: Colon; L3: Ileocolon; L4: Upper gastrointestinal location; E1: Ulcerative proctitis; E2: Left sided UC; E3: Extensive UC; NA: Not-applicable; NS: Not significant; CDAI: Crohn's disease activity index. Systemic steroids included: Methylprednisolone and prednisone; topical steroids included: Budesonide and beclomethasone dipropionate; biological agents included: Infliximab and adalimumab.

Table 2 Mucosal viral loads

	HCMV	EBV
Number of patients	15/80	32/80
Refractory	286 (0-221.697)	5.440 (0-966.333)
Diseased mucosa	30.763 (11.911-221.697)	8.294 (1.020-966.333)
Non-diseased mucosa	0 (0-3)	281 (12-400)
Responders	0 (0-273)	6 (0-973)
Diseased mucosa	0 (0-273)	9 (0-973)
Non-diseased mucosa	0 (0-2)	4 (0-63)
Controls	0 (0-41)	0 (0-34)

The values are given as copies of DNA/10⁵ cells and showed as median (range). HCMV: Human Cytomegalovirus; EBV: Epstein-Barr virus.

non-refractory group, the hospitalization rate was 39%.

Mucosal viral load

Detectable copies of viral DNA were found in all (17/17, 100%) refractory, 13 out of 23 (56.5%) non-refractory IBD patients, and 13 out of 40 (32.5%) controls, with HCMV DNA evident in four refractory and five non-refractory IBD patients, and two controls, EBV DNA found in nine refractory and eight non-refractory IBD

patients, and 11 controls, and double positivity found only in four refractory patients. The median values with ranges of DNA copies of both viruses, as obtained by pooling together the data from all colonic segments of each patient and then, in the IBD groups, from both inflamed and non-inflamed mucosa, are shown in Table 2. It is worth of noting that the median values for both HCMV (Figure 1, panel A) and EBV (Figure 1, panel B) were significantly higher in refractory IBD compared to non-refractory IBD patients and controls, while non-refractory IBD patients did not show significantly different values compared to controls (Figure 1, panels A and B). Moreover, refractory IBD patients invariably showed DNA peak values $\geq 10^3$ copies/10⁵ cells in at least one colonic segment, while patients with non-refractory IBD and controls displayed viral DNA peak levels below 10³ and 10² copies/10⁵ cells, respectively (Figure 1, panels A and B). Finally, upon analyzing the viral DNA loads within the refractory IBD group, the median values were found to be significantly higher in diseased vs non-diseased mucosa (Figure 1, panels C and D), whilst no difference was found when the median viral DNA levels of non-diseased mucosa were compared with those found in both non-refractory IBD patients and controls. It is worth noting that there was a positive correlation ($r = 0.71$ and 0.79 for HCMV and EBV, respectively) between the mucosal viral load in the refractory IBD and the degree of endoscopic activity (Table 1). In this regard, a narrow overlap between the extension and severity of mucosal le-

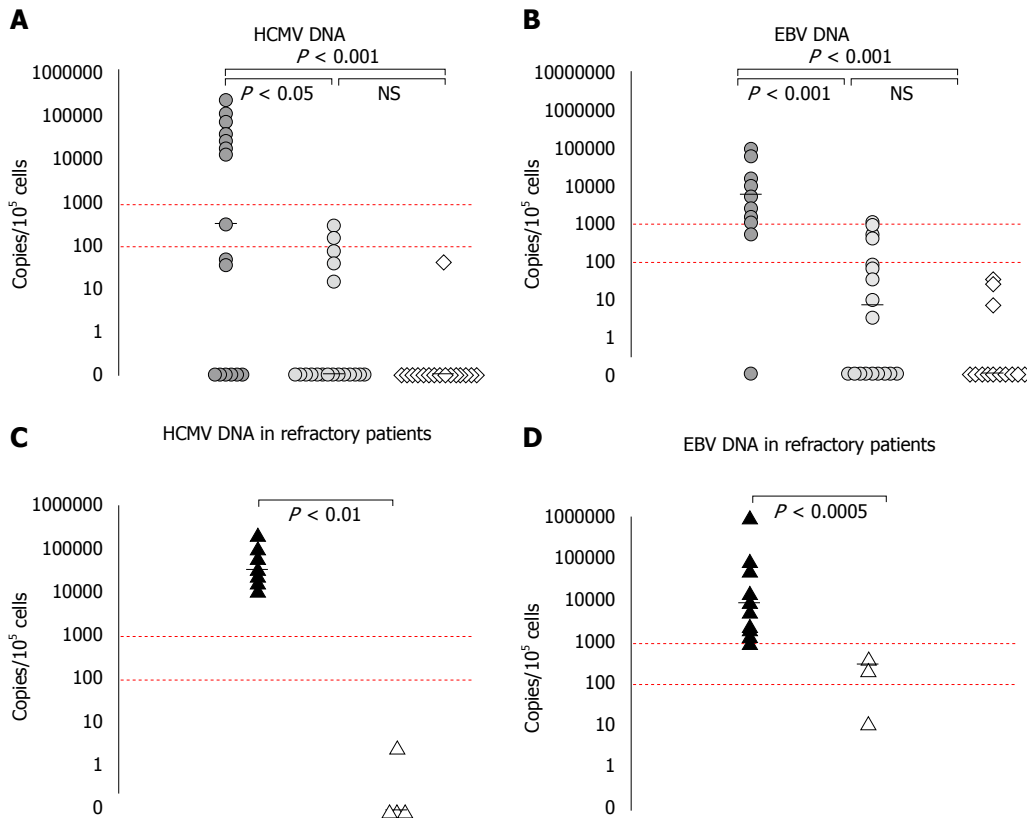


Figure 1 Refractory inflammatory bowel disease patients (full circles) showed statistically significant higher values in comparison with non-refractory patients (empty circles) and controls (empty rhombuses; panels A and B). A peculiar distribution is observed with non-refractory patients displaying values always below 10^3 and controls below 10^2 copies/ 10^5 cells (red dotted lines). Within the refractory group, the macroscopically diseased areas (full triangles) carried DNA viral loads invariably over 10^3 copies/ 10^5 cells compared to non-diseased mucosa (empty triangles; panels C and D). The black bars indicate the median values. NS: Not significant; HCMV: Human Cytomegalovirus; EBV: Epstein-Barr virus.

sions with the viral load distribution was invariably found (representative cases are shown in Figure 2). By contrast, no correlation between viral load and clinical indexes of disease activity and no preferential association between HCMV or EBV with UC or CD were observed.

Blood viral load

Viral DNA was detected in 11/17 patients with refractory IBD (median values for HCMV and EBV: 0 copies/mL, range: 300–26,000, and 100–7,900, respectively), 1/23 of non-refractory IBD (450 copies/mL), and none of the controls. Specifically, two refractory patients showed HCMV DNA, five refractory patients and one non-refractory patient had EBV DNA, and four refractory patients carried both DNAs. All these patients carried the same virus(es) at mucosal level, and a significant positive correlation between the values in the two compartments ($r = 0.67$ for HCMV and 0.61 for EBV) was observed. The sensitivity of the PCR assay performed on peripheral blood samples as compared to that on tissue samples, therefore, was 23% for HCMV and 45% for EBV, with a specificity of 100% for both, while the positive predictive value was 76.4% and 17.6%, and the negative predictive value was 65.0% and 80.9% for HCMV and EBV, respectively.

Histological and endoscopic features

As far as immunohistochemistry is concerned (representative cases shown in Figure 3), although the specimens were harvested from the same mucosal areas as those taken for the PCR assay, HCMV positive cells were found in only 11 cases (five had a DNA load $> 10^3$ copies/ 10^5 cells, and six were negative), while EBV positive cells were detected in 17 cases (five had a viral DNA load $> 10^3$ copies, eight $< 10^3$ copies/ 10^5 cells, and four negative), thus showing a sensitivity of 33% for both viruses, a specificity of 90% for HCMV and 0% for EBV, with positive predictive values of 66% and 80%, and negative predictive values of 71% and 0% for HCMV and EBV, respectively. No correlations between the mean number of positive cells and the level of mucosal viral load for either virus or the degree of endoscopic activity were found.

As regards the endoscopic features of refractory IBD patients, the mucosal lesions appeared indistinguishable from those of the underlying disease, despite the invariable presence of the superimposed viral end-organ disease, as evident in Figures 4, 5, and 6 showing representative cases. It is worth noting that mucosal healing was observed in all cases with isolated HCMV colitis (Figure 4, panel C and F) following specific antiviral therapy (see below). Conversely, no amelioration was evident in any of the cases carrying

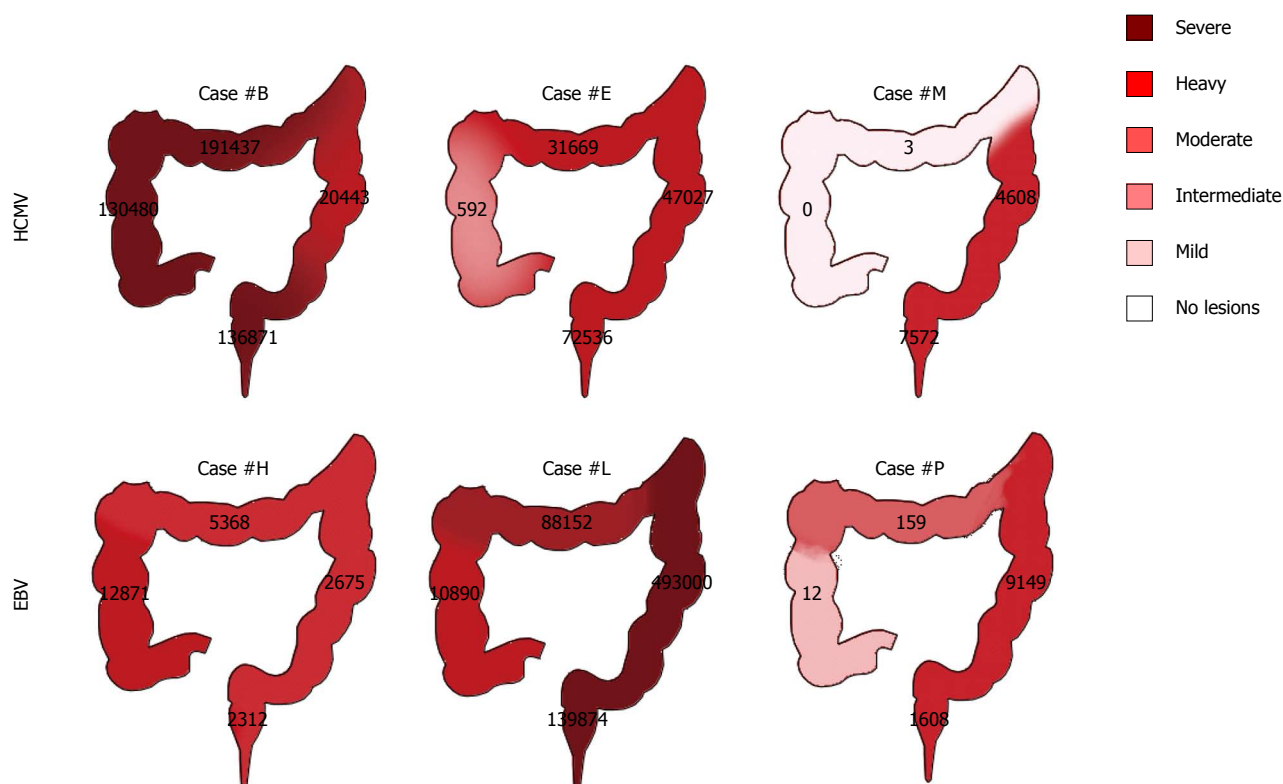


Figure 2 Six representative refractory patients with Human Cytomegalovirus - upper panels - and Epstein-Barr virus - lower panels - superimposed colitis. The distribution of the DNA viral loads (given as number of copies/ 10^5 cells) along the colon perfectly matches the distribution and severity of mucosal lesions as shown by the arbitrary red scale. HCMV: Human Cytomegalovirus; EBV: Epstein-Barr virus.

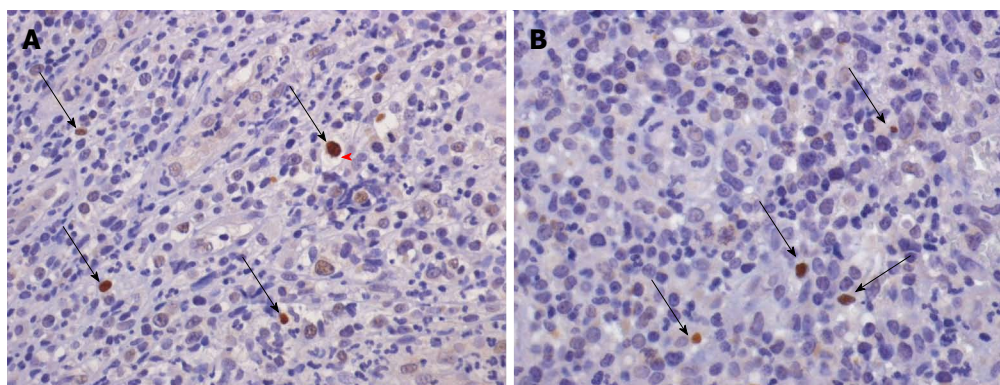


Figure 3 Pathognomonic "cytomegalic" cell (*i.e.*, enlarged cell surrounded by a light-coloured halo, red arrow) with a brown reactive nuclear inclusion (thin black arrows) and a few scattered positive cells for the human Cytomegalovirus are shown (panel A, immunoperoxidase-hematoxylin, original magnification $\times 250$). Some positive cells with brown nuclei (thin black arrows) are evident following the specific staining for the Epstein-Barr virus nuclear antigen-1 (panel B, immunoperoxidase-hematoxylin, original magnification $\times 400$).

both viruses (Figure 6, panels C and F), nor in most of those with EBV (Figure 5, panels E and F), except the patient (Figure 5, panel D) who underwent a course of treatment with rituximab (see below).

Impact of current therapies

Systemic steroid use was identified as a significant risk factor for both HCMV and EBV colitis ($P = 0.018$ and 0.0020 , OR = 11.4 and 12, 95%CI: 1.23-106.11 and 2.37-60.67, respectively), while the use of biologic agents and topical steroids was closely related to EBV

colitis ($P = 0.021$ and 0.008 , OR = 7.8 and 10.2, 95%CI: 1.32-46.64 and 1.73-60.92, respectively). No significant correlation was found between an increased risk of development of viral end-organ disease and the use of immunosuppressants, the duration of both therapies and underlying disease, age, gender, smoking habits, or body mass index.

Patients' outcome

All refractory patients with HCMV colitis underwent a course of specific antiviral therapy with ganciclovir

Endoscopic features of HCMV colitis

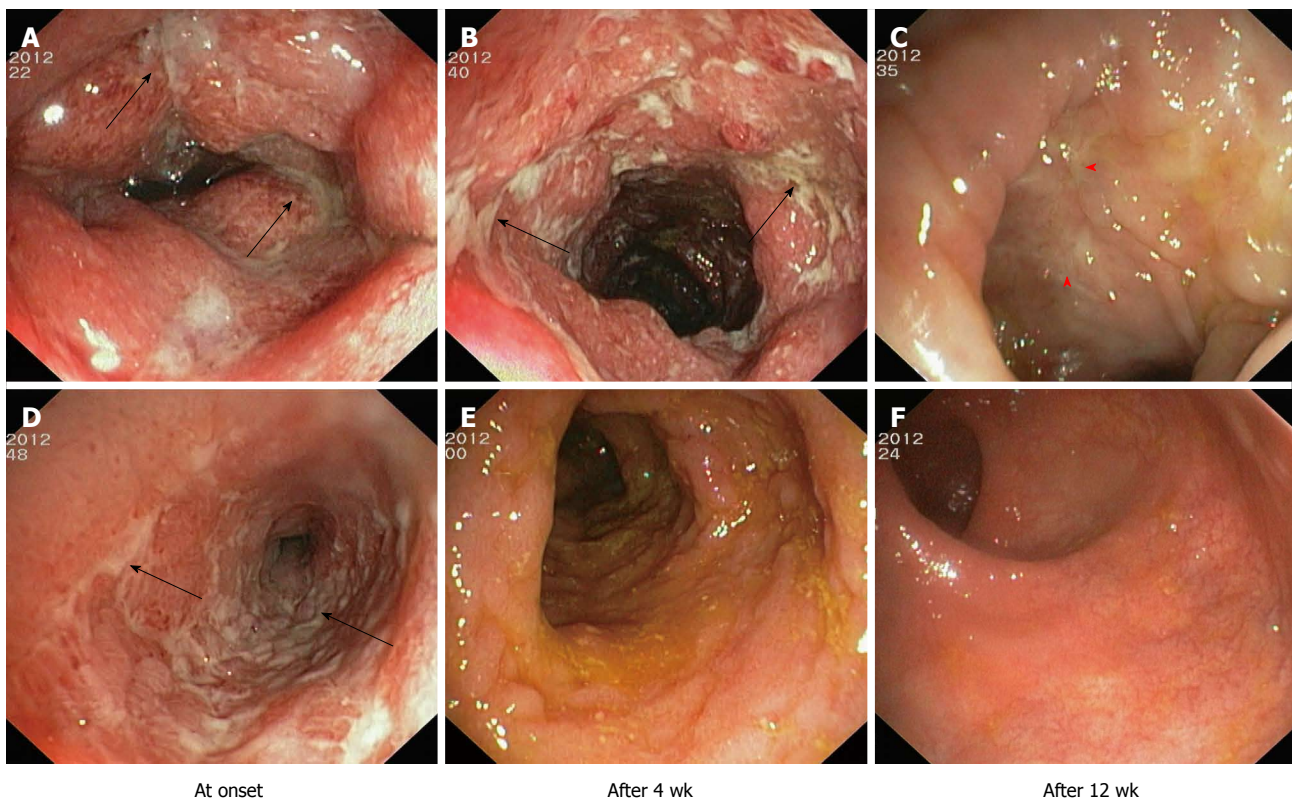


Figure 4 Presence of profound, round and longitudinal ulcers covered by a fibrino-purulent exudate (black arrows) and embedded in edematous and erythematous mucosa are clearly evident (panels A and D). The healing process, usually observed after three months from the end of specific antiviral therapy, may result in both white scars (red arrows, panel C) or restitution ad integrum (panel F), passing through a phase of slight improvement (panels B and E).

Endoscopic features of EBV colitis

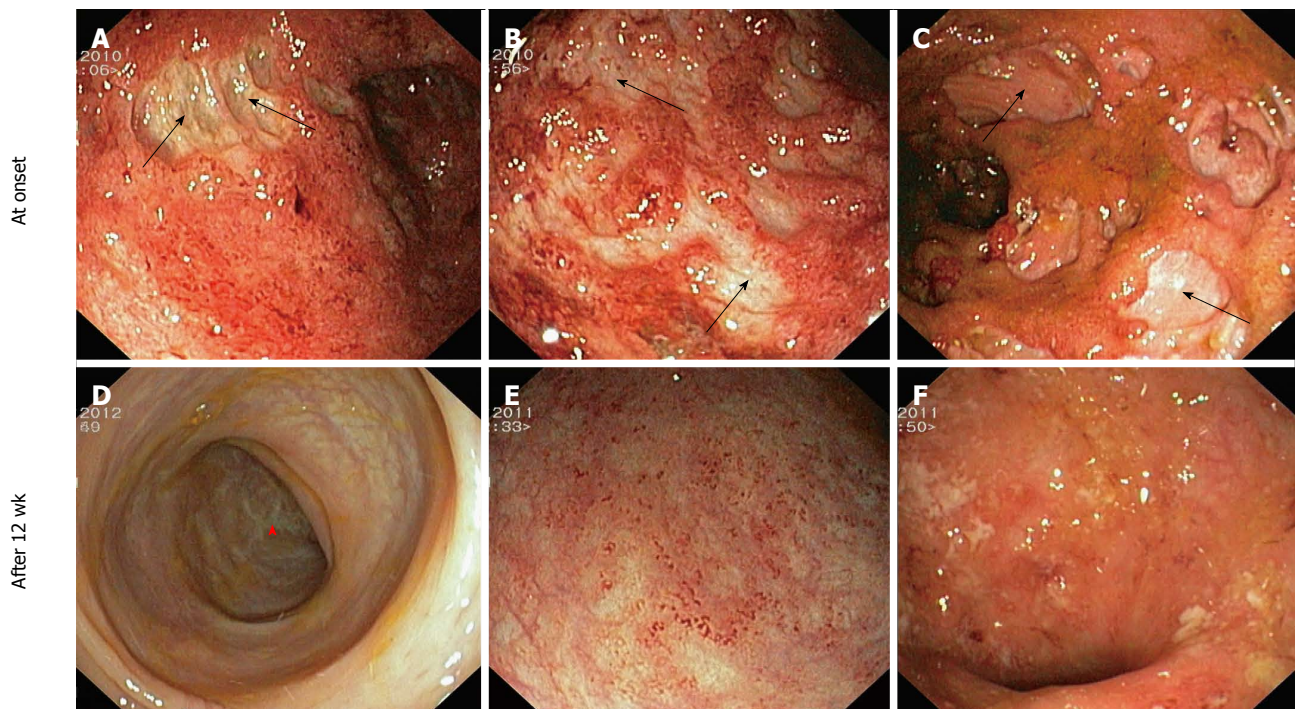


Figure 5 Presence of holes in the mucosa with exposure of the underlying muscular layer (black arrows), surrounded by granular and spontaneously bleeding zones, is clearly evident (panels A to C). After a three-month washout period from any therapy for the primary disease, the healing process with white scars (red arrow) was observed in the only patient who underwent a cycle with rituximab (panel D), whilst a slight or no improvement was observed in the other two representative cases.

Endoscopic features of EBV plus HCMV colitis

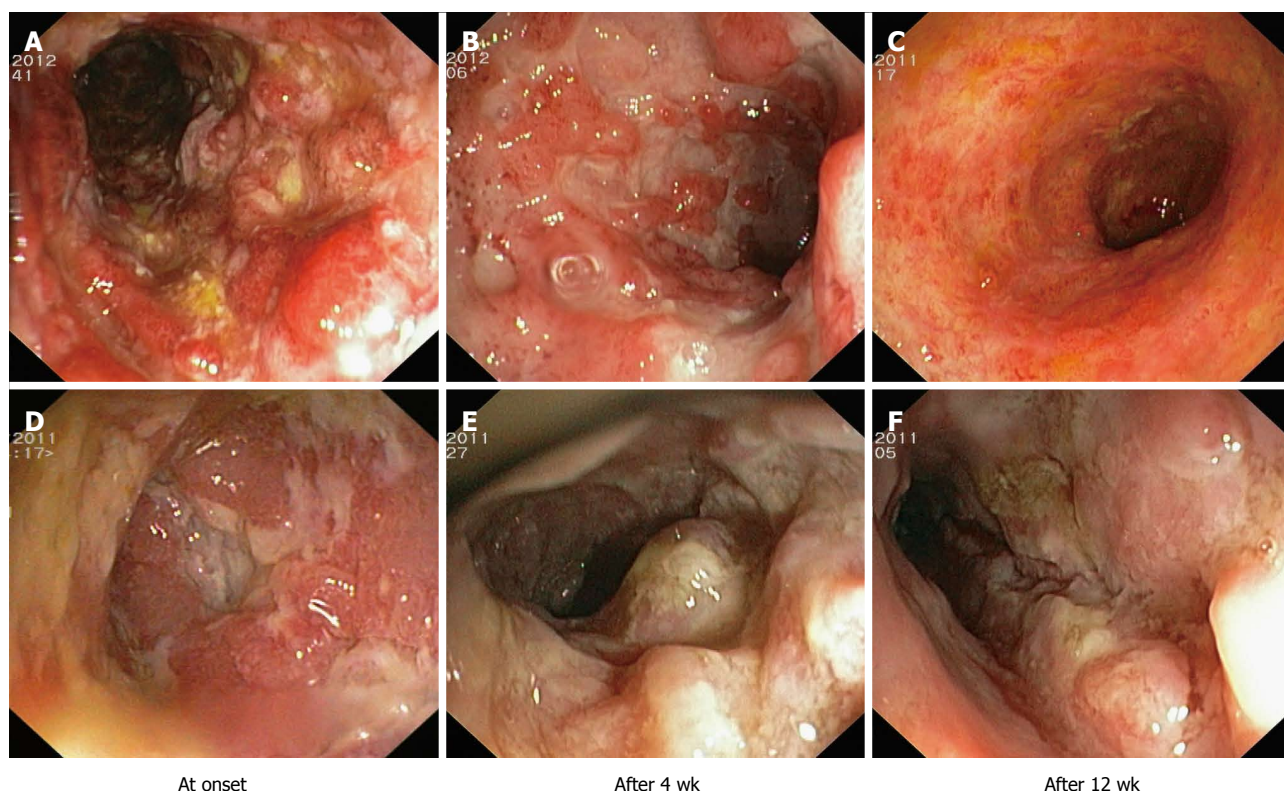


Figure 6 Severe lesions characterized by a pronounced, nodular-cobblestone appearance, punctuated by multiple, deep ulcerations (panels A, B, D, E), which did not heal (panels C, F), were found in those refractory patients with high DNA loads of both viruses.

(5 mg/kg bid iv) for three weeks^[11], with monitoring of mucosal and blood viral load at the end of therapy and, following discharge, after four and 12 wk, as arbitrarily scheduled. All patients reached a satisfactory general condition with a sharp decrease in both clinical and endoscopic indexes of activity [median values: 249 (range: 166-302) and 10.37 (8.7-12.2) after four weeks, and 173 (132-209) and 7.2 (5.9-9.3) after 12 wk for CD patients, and 7 (range: 4-9) and 1 (2-1) after four weeks, and 5 (4-7) and 1 (1-2) after 12 wk for UC patients]. The viral mucosal DNA levels also decreased significantly ($P = 0.031$) by the end of therapy (Figure 7A), and remained stable afterwards (data not shown), with patients experiencing a good quality of life during a median follow-up period of 11 mo (range: 6-22). In contrast, refractory patients with EBV colitis did not show any improvement in either clinical or endoscopic activity indexes [median values: 396 (range: 281-595) and 11.49 (range: 8.9-12.4) after four weeks, and 295 (range: 351-244), and 10.44 (range: 9.0-11.7) after 12 wk for CD patients, and 11 (range: 8-13) and 2 (range: 2-3) after four weeks, and 11 (range: 8-12) and 2 (range: 2-3) after 12 wk for UC patients]. Likewise, no significant decrease of mucosal (or blood when positive) EBV DNA levels was observed, despite steroid tapering and discontinuation of the other medications in 9/15 patients (Figure 7B). Remarkably, in the only case where the patient

underwent treatment with the anti-CD20 monoclonal antibody rituximab (375 mg/m² body surface iv weekly for 4 wk^[8]), EBV DNA was cleared from both blood and colonic mucosa. However, after six months, a worsening of the patient's clinical condition characterized by fever and intestinal bleeding was observed, with mucosal EBV load $> 10^3$ copies/10⁵ cells, resulting in a colectomy. In this regard, 7/9 refractory patients with EBV colitis underwent colectomy within a median period of 5 mo (range: 2-9), as well as 3/4 patients carrying both virus DNAs within a median period of 8 mo (range: 5-14), despite the fact that HCMV had been cleared by antiviral therapy. The remaining patients developed chronically active colitis, thus adversely affecting their quality of life. During the same follow-up period, only two non-refractory CD patients underwent intestinal resection due to strictures.

DISCUSSION

Despite a growing interest in opportunistic viral infections in IBD, several crucial points still remain unsolved, such as their prevalence and role in tissue damage during exacerbation, the best diagnostic and therapeutic approaches, as well as the risk factors^[2,3,10,11]. The reported frequency of HCMV infection, for instance, ranged from 10%^[23,24] to 36%^[25,26], whilst that of EBV was higher, ranging from 41%^[27]

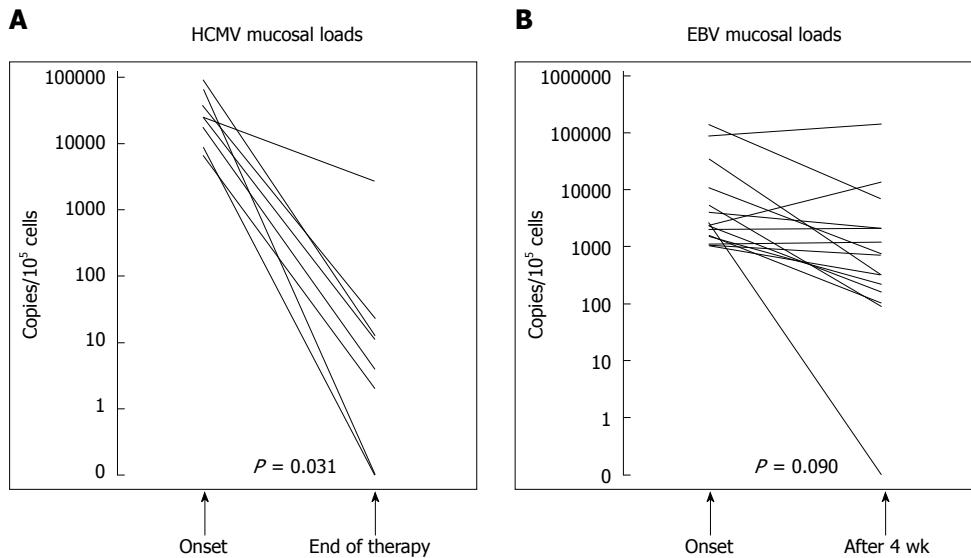


Figure 7 Median values of human Cytomegalovirus (panel A) and Epstein Barr virus (panel B) DNA levels of each refractory patient at onset and at the end of antiviral therapy in human Cytomegalovirus cases, and at four weeks after washout or reduction of current treatment in patients with Epstein-Barr virus infection. A dramatic decrease of mucosal viral loads was observed in all patients with HCMV colitis, except one who continued steroid therapy while taking ganciclovir. By contrast, no modification of mucosal viral load values was observed in any patient with EBV colitis, except the one who underwent a cycle of therapy with rituximab. HCMV: Human Cytomegalovirus; EBV: Epstein Barr virus.

to 64%^[28]. In addition, most of the studies were retrospective, included surgery specimens, applied different techniques, and therefore, the results are not comparable. As far as the detection method is concerned, immunohistochemistry was the most widely used method^[23,28-30], and it is given as the screening test in the decisional algorithm for the management of HCMV infection in IBD^[31]. However, quantitative real-time PCR assay carried out on nucleic acids extracted from formalin-fixed paraffin-embedded intestinal specimens has recently proved to increase the sensitivity of immunohistochemistry^[32]. Moreover, the application of this technique on fresh biological samples has emerged as the best technique^[24,33-35] as it has the advantage of being highly sensitive, rapid, and reproducible. Our results fit in with this evidence, since when comparing the two techniques, immunohistochemistry showed low predictable values, both positive and negative, possibly due to the suboptimal specificity and sensitivity of the primary antibodies used and the lack of correlation with the lytic phase of the viruses. Similar results were obtained in congenital HCMV infection when the same primary antibodies were used^[36], which hamper its usefulness in the management of IBD patients. By contrast, quantitative real-time PCR on freshly collected mucosal biopsies displayed not only a better performance, but also allowed us to distinguish between viral infection and colitis, since a positive result did not necessarily imply that the patient was suffering from an active HCMV or EBV end-organ disease. Accordingly, two main groups of mucosal viral load may be identified: that with peak values greater than 10^3 copies/ 10^5 cells, and that with values below this threshold. Since all refractory patients

carried mucosal viral loads invariably greater than 10^3 copies/ 10^5 cells in at least in one colonic segment, it is conceivable that this indicates a symptomatic viral-related colitis that is superimposed on the underlying primary illness. Interestingly, our systematic sampling of all colonic segments made it possible to build up a map of the mucosal viral loads that perfectly overlaps with the severity of macroscopic lesions and correlates with the endoscopic indexes of activity. On the other hand, the presence of a number of DNA copies lower than 10^3 / 10^5 cells only in patients with quiescent disease might be related to the periodic viral reactivation that usually occurs even in healthy people without this triggers of symptoms^[4]. However, on comparing the values found in non-refractory IBD patients with those found in control patients, a median mucosal peak value of 1 logarithm unit higher was observed in IBD patients. This finding could prove to be of some clinical use as a warning for closer follow-up and caution in prescribing those therapies that favor the disruption of the delicate balance between the host immune response and viral activity, which leads to precipitation or exacerbation of mucosal injury. A similar distinction between viral colitis and infection could not be achieved by previous studies even when applying PCR-based techniques, since the differences in expressing the results (as copies by microgram of total DNA extracted^[24,34] or milligram of tissue^[33]), the sensitivity of the test, and the lack of control population made it impossible to draw definite conclusions. In this regard, the possibility of measuring the DNA viral load in stool samples, thus avoiding an invasive endoscopic exam, has already been explored^[37]. However, the lack of control populations, the limited number of cases enrolled and,

in most of all, the absence of an exact correspondence between the levels found in the mucosa and stool samples, did not allow the authors in question to differentiate between viral end-organ disease and reactivation/infection. Moreover, the presence of some technical limitations such as the need for a fresh, liquid stool sample made this diagnostic test unsuitable for the management of IBD patients. Finally, the search for circulating class M specific anti-virus antibodies is rather ineffective in detecting an active disease, since elevated levels can persist for up to two years after infection, and immunocompromised patients may not mount an IgM response^[38].

We believe, therefore, that the previous definitions of HCMV or EBV infection and colitis, often used interchangeably, and simply referring to the evidence of viral positivity in colonic tissue of symptomatic patients by means of any technique^[11,38], are confusing and should be assessed by applying the quantitative real-time PCR method on freshly harvested mucosal biopsies. However, further studies are warranted in order to optimize the mucosal sampling and assess the risk stratification in order to achieve early recognition and appropriate management of these conditions. It is worth noting that we confirmed^[39,40] EBV as the most prevalent infection, since half (49%) of the IBD patients and almost all of the refractory patients (88%) showed EBV positivity alone or in combination with HCMV. This observation is of great clinical relevance if we consider that, so far, the majority of papers have instead focused on HCMV infection. Furthermore, the exact correspondence between the macroscopically damaged areas and those carrying the highest viral loads strengthens the hypothesis that both viruses play a role in contributing to mucosal lesions. In this regard, a suggested viral tropism to sites of inflammation^[41], and their ability to affect cytokine production may account for how they escape host immune surveillance directly at mucosal level^[42]. Moreover, the possible presence of malnourishment and the increasing and early use of aggressive therapies^[38] whose main target are T-cells, which play a crucial role in controlling HCMV and EBV latency and reactivation^[43], represent potential co-factors in triggering viral colitis. The use of steroids, azathioprine or 6-mercaptopurine, and infliximab, indeed, has been found to produce similar effects in significantly increasing the OR for HCMV infection (3.4, 3.1, and 4.4, respectively), with the combined use of two or three of these drugs yielding an OR of 14.5^[2,44]. However, infliximab did not appear to affect the incidence of latent virus reactivation in the short-term^[45], but mostly in long-term treatment^[3]. Our results only partially confirmed these data, since immunosuppressants did not increase the risk of developing a superimposed viral colitis, while the use of biologic agents and topical steroids was closely related to EBV colitis. Moreover, although anti-tumor necrosis factor- α agents have been shown to cause widespread HCMV infection^[46], we did not substantiate

this result. Most importantly, as in transplant patients^[47], systemic steroid use was identified as a significant risk factor for both HCMV and EBV colitis, probably due to their ability to increase viral protein production, as shown *in vitro*^[48]. No additional factors, including duration of therapy or disease, age and degree of malnourishment were found to be related to the risk of developing viral colitis. Interestingly, only those patients with mucosal viral loads approaching 10^5 copies/ 10^5 cells showed detectable viral DNA even in peripheral blood, as already found in recipients of solid organ transplants where a HCMV end-organ disease may exist independently from systemic involvement^[49].

Finally, if our evidence is confirmed by larger studies, the rate of "true" refractoriness to standard therapies may shrink considerably, since a sizeable number of patients would instead be diagnosed as suffering from viral colitis. Remarkably, mucosal healing, mirrored by a sharp decrease in the viral load, was invariably observed in HCMV colitis following specific antiviral therapy^[11] together with a quick tapering and then discontinuation of steroids, whilst the immunosuppressive and biological agents may be continued by virtue of their long-lasting effect, which blocks any attempt to recover immunological competence in the short term. By contrast, both the mucosal viral load and indexes of activity remained largely unmodified in those patients with EBV colitis, despite the washout or tapering of the current therapies, and the vast majority of them underwent colectomy. The only patient who showed a substantial, although transient, drop in the mucosal viral load was the one treated with rituximab in an attempt to rapidly deplete the B lymphocytes which host the virus^[41], since no efficacious therapy is available to date^[4].

In conclusion, the use of quantitative real-time PCR assay on freshly harvested mucosal specimens proved to be more effective than immunohistochemistry in the diagnosis of opportunistic viral infection and allowed us to identify a threshold value that distinguishes infection from end-organ disease, whose clinical management is largely different.

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COMMENTS

Background

Inflammatory bowel disease (IBD), including Crohn's disease and ulcerative colitis, are disabling, lifelong, pathological conditions affecting the gastrointestinal tract, mainly the colon, triggered and sustained by a dysregulated immune response towards antigens of the gut microbiota. A better understanding of the fine mechanisms responsible for tissue injury has led to the use of more aggressive therapies even in the early phase of the disease aimed at achieving mucosal healing and preventing disease progression. However, the increasing use of immune-suppressant and immune-modulant molecules carries the risk of

opportunistic infections, including those due to human Cytomegalovirus (HCMV) and Epstein Barr virus (EBV).

Research frontiers

To date, the information available on the frequency, role and risk factors of HCMV and EBV infections in IBD exacerbation and their diagnostic and therapeutic approaches is conflicting. The causes for this discrepancy lie in the differences amongst the patients enrolled, the diagnostic methods applied, and the retrospective design of the majority of studies.

Innovations and breakthroughs

This study prospectively investigated the presence of HCMV and EBV infection in IBD patients and control subjects by applying immunohistochemistry and quantitative real-time polymerase chain reaction (PCR) assay, carried out on both peripheral blood and fresh mucosal samples. The latter proved to be the best diagnostic technique, since it allowed us to distinguish between viral colitis and infection by identifying a cutoff value. Interestingly, all refractory IBD patients carried the highest mucosal viral loads, which correlated with the severity of mucosal damage and endoscopic activity. EBV infection was found to be the most prevalent, and steroid therapy was identified as a significant risk factor. Finally, with a view to treatment, a course of antiviral therapy was of benefit in determining both the disappearance of viral DNA and mucosal healing in HCMV-related colitis, whilst the vast majority of patients with EBV colitis underwent colectomy.

Applications

This study contributes to our understanding of the frequency and role of opportunistic viral infection in refractory IBD patients and provides the basis for the use of real time quantitative PCR as the gold standard diagnostic technique in differentiating viral end-organ disease from infection. This technique also allows the patients to be monitored.

Terminology

Inflammatory bowel diseases are chronic enteropathies triggered and sustained by an abnormal immune response to usually-tolerated antigens, which develops in genetically susceptible individuals. Refractoriness is defined as the lack of response to current therapies. Opportunistic viral infections, including HCMV and EBV, are those caused by organisms capable of establishing latency in target cells and reactivating in cases of reduced host defence, such as during immunosuppressive or immunomodulatory therapy, giving rise to both systemic and end-organ disease, which can also be localized to the gastrointestinal tract.

Peer-review

The authors investigated CMV and EBV in tissue specimens of refractory and non-refractory mixed IBD patients by quantitative real-time PCR and immunohistochemistry. Additionally, the whole colon was mapped in order to correlated viral loads to endoscopic lesions. This is a very good paper.

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