Increased state of activation of CD4 positive T cells and elevated interferon γ production in pouchitis

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Abstract

Background—Immunoregulatory abnormalities of T cells might be of importance in the pathogenesis of pouchitis after ileoanal pouch anastomosis (IAP).

Aims—To characterise T cell subsets, their state of activation, and production of cytokines in inflamed and non-inflamed pouches in patients with ulcerative colitis (UC) and familial adenomatous polyposis (FAP). The influence of T cell activation on mucosal transformation was also studied.

Patients—Mucosal biopsy specimens were taken from 42 patients with IAP (33 with UC and nine with FAP).

Methods—Mononuclear cells were isolated by standard techniques and characterised by three colour flow cytometry. Interferon γ (IFN- γ) production was studied using the ELISPOT technique.

Results—In patients with UC with pouchitis there was a significant increase in the CD4:CD8 ratio, expression of activation markers on CD3+ cells, and number of IFN γ producing mononuclear cells compared with patients with UC without pouchitis (CD4:CD8 ratio 1.3 (range 0.7–2.7) versus 0.6 (0.1–1.0), $p=0.012$). In addition, a positive correlation between increased crypt depth and the number of CD4+ cells ($r=0.57$) was shown.

Conclusion—The observed increase in activated mucosal CD4+ T cells and IFN- γ production might lead to mucosal destruction and crypt hyperplasia as seen in pouchitis.

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Keywords: pouchitis; T cell activation; mucosal transformation

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reemergence of UC. Pouchitis is mainly seen in patients with UC; pouchitis is very rarely described in FAP patients.⁶ Patients with extensive colitis are more likely to develop inflammatory changes in their reservoirs than those with more limited disease of the colon.⁷ In addition, similarities in distribution of immunoregulatory cells and cytokine pattern in pouchitis and UC have been reported.^{8–10}

Our knowledge of immunological changes in the pouch mucosa is very limited compared with studies in chronic inflammatory bowel disease (IBD) mucosa. In the intestinal mucosa of patients with IBD there are no major differences in the phenotype of intestinal T cells as shown by immunohistological and cytofluorometric studies.¹¹ However, in studies investigating the state of activation of mucosal lymphocytes in patients with IBD it has been shown that the number of mononuclear cells expressing CD25 or other activation markers is increased in affected areas compared with unaffected areas or control tissue.^{12–14} Another important finding is an enhanced responsiveness to T cell receptor stimulation in IBD compared with controls.^{15–16} These and other studies show an increased state of T cell activation in inflammatory lesions and indicate an up regulated mucosal immune response in patients with IBD (for review see Zeitz and Stallmach¹⁷). As a consequence of T cell activation cytokines are released that influence the activity, differentiation, and proliferation of a variety of cells and mediate tissue injury. It is well established that proinflammatory cytokines such as IL-1- β or tumour necrosis factor α (TNF- α) are released to a greater extent in the inflamed mucosa by macrophages/monocytes and are considered to be involved in secondary pathophysiological mechanisms such as tissue damage observed in pouchitis.^{18–19} However, little is known about the concentration of immunoregulatory cytokines such as interleukin 2 (IL-2) and IFN- γ (Th1-like cytokines) and IL-4 and IL-10 (Th2 like cytokines) in the intestinal mucosa of patients with IAP.

Mucosal biopsy specimens from functioning pouches after IAP commonly show villous atrophy, crypt hyperplasia, and a change in mucin type from small bowel sialomucin to a large bowel type sulphomucin. These chronic inflammatory changes in the pouch resemble mucosal transformation of the hyper-regenerative type and can be interpreted as an adaptation of the ileal mucosa to luminal

Restorative proctocolectomy with ileoanal pouch anastomosis (IAP) is an established surgical procedure for the management of ulcerative colitis (UC) and familial adenomatous polyposis (FAP).^{1–2} Pouchitis is a well known complication following restorative proctocolectomy. The clinical entity of pouchitis was first described in continent ileostomies for UC and later in pelvic ileal pouches.^{3–4} Pouchitis is most common in patients with UC and an incidence of 7–42% has been reported recently. The aetiology and pathogenesis of pouchitis are unknown.⁵ There are multiple observations suggesting that pouchitis might represent a

Table 1 Clinical characteristics

	Ulcerative colitis with pouchitis	Ulcerative colitis without pouchitis	Familial adenomatous polyposis
No of patients	14	19	9
Age (y)	38.5 (20–43)	38 (18–56)	37 (19–60)
Sex ratio (M:F)	8:6	11:8	5:4
Disease duration (months)	112 (38–192)	144 (38–228)	
Pouch age (months)	25 (2–87)	12 (2–47)	48 (7–74)
PDAI	9 (7–15)	2 (1–5)	2.5 (0–4)

changes.⁹ Acute inflammation of the pouch (pouchitis) is reflected by macroscopic lesions as erythema, haemorrhages, focal ulcerations, aphthous lesions, and total villous atrophy. Experimental studies on fetal intestinal explants have shown similar changes which were induced by activation of mucosal T cells.^{20–22} The sequential follow up of immunological changes in the pouch mucosa after IAP may thus provide a clinical model to study the relation between the predominance of certain T cell subsets, T cell activation, cytokine production, and mucosal transformation or destruction. In addition, these investigations may provide insight into the pathogenesis of UC.

Methods

PATIENTS

Mucosal biopsy specimens were obtained from ileoanal pouches and the ileum proximal to the anastomosis from 19 patients with UC with a non-inflamed ileoanal pouch and 14 patients with UC with clinical, endoscopic, and histological signs of pouchitis. The Pouchitis Disease Activity Index (PDAI) described by Sandborn was equal or higher than 7 points in all patients (median 9, range 7–15).⁵ Follow up studies were done in six patients with acute pouchitis, who were treated with metronidazole (1200 mg/day orally) for two weeks. Data for these patients were compared intraindividually after improvement of pouchitis.

All patients had a total proctocolectomy with the creation of an ileoanal J pouch by the same team of surgeons (K-WE, GF) by a one or two stage procedure. Nine patients who had an ileoanal pouch after colectomy for FAP served as controls (table 1). Simultaneous blood samples were taken from all patients. The study was approved by the local ethics committee.

PREPARATION OF MUCOSAL AND PERIPHERAL BLOOD MONONUCLEAR CELLS

Endoscopic biopsy specimens from patients with ileoanal pouches were obtained during routine endoscopy using a small diameter endoscope (Olympus PQ10). Mucosal mononuclear cells were isolated as described previously.²³ Briefly, seven to 10 biopsy specimens were minced and incubated overnight on an orbital shaker at 37°C in RPMI 1640 medium containing 10% fetal calf serum, 25 mM HEPES buffer, 0.05M 2-ME, 100 U/ml penicillin, 100 µg/ml streptomycin, 0.01% collagenase (CLS III, Worthington), 0.01% deoxyribonuclease (Boehringer, Mannheim, Germany), and 0.01% soybean trypsin inhibitor (Sigma, Deisenhofen, Germany). The suspension was passed through a stainless sieve, and cells were washed once in 30% isotonic Percoll

solution (Pharmacia, Uppsala, Sweden) to remove cell debris and centrifuged on a Ficoll-Hypaque density gradient. Viability of the resulting lymphocyte population determined by trypan blue dye exclusion was always more than 90%. Peripheral blood mononuclear cells were isolated by Ficoll-Hypaque density gradient centrifugation.

IMMUNOFLUORESCENCE STUDIES

Mucosal lymphocytes were stained with saturating concentrations of various combinations of the following directly FITC, PE, and rhodamine-PE-Cy5 conjugated monoclonal antibodies: CD3 (Immunotech, Marseille, France), CD4, CD8, CD25, HLA-DR, and CD45R0 (all Dako, Hamburg, Germany). Isotype matched mouse monoclonal antibody not reactive with human leucocytes was used as control (Dako, Germany, FRG). After staining cells were fixed for 30 minutes with 1% paraformaldehyde and 1×10^5 cells were analysed by three colour laser flow cytometry (FACSVantage, Becton Dickinson), using CellQuest software provided by Becton Dickinson. Lymphocyte populations were gated by forward/sideward scatter analysis to exclude monocytes. An electronic gate was added to include only CD3+ T cells for further analysis.

ELISPOT ASSAY

The ELISPOT assay is based on immune-enzyme technology originally developed for the enumeration of antibody secreting cells.²⁴ The same principle has been used for the development of the ELISPOT assay detecting INF- γ producing cells.²⁵ In brief, 1×10^5 lamina propria mononuclear cells (LPMNCs) were added to the wells of a microtitre plate (Hözl, Cologne, Germany; cat. no. E 914) coated with mouse monoclonal IgG1 antibodies specific for INF- γ . Cells were incubated for 24 hours in the presence or absence of phytohaemagglutinin (PHA, 2 µg/ml). After incubation, cells were removed by extensive washing and the wells were filled with a dilution of second mouse monoclonal anti-INF- γ antibody, which binds to a different epitope. Subsequently, a goat antirabbit IgG alkaline phosphatase conjugate was added and after an additional washing step all wells were filled with a chromogen substrate. After colour development, spots could be counted by light microscopy. Results were expressed as INF- γ producing cells per 1×10^5 LPMNCs.

MORPHOMETRY

The ileal mucosa was examined morphometrically as described by Clarke.²⁶ Biopsy specimens were fixed in ethanol/glacial acetic acid (3:1, vol/vol) for 24 hours, then rinsed with and stored in 75% ethanol. Specimens were stained with the Feulgen reaction before the individual villi were prepared under a stereomicroscope. From each sample 10 random isolated villi were chosen for measurement of their height, depth, and width, at both the base and apex. From these values villous surface area was calculated. Subsequently, crypts were dissected and their depths recorded. The medians were

Figure 1 Representative example of flow cytometric analysis of CD4 and CD8 expression on CD3+ T cells on mucosal lymphocytes. Lymphocytes were isolated from inflamed pouch mucosa (A) and non-inflamed mucosa (B) of one patient with UC after treatment with metronidazole.

calculated from the morphometric data for each specimen. Values were measured by two independent observers (AS and SH) and inter-observer results varied by less than 10%.

STATISTICAL ANALYSIS

Results were described as means and standard deviations or, if values were not distributed normally as medians and ranges. Statistical significance was calculated using the *t* test or Wilcoxon rank test, and considered to be significant at the $p \leq 0.05$ level. Spearman rank

correlation coefficients were calculated using the StatView SE+Graphics program on a Macintosh computer.

Results

EXPRESSION OF CD4 AND CD8 ON MUCOSAL LYMPHOCYTES AND PERIPHERAL BLOOD LYMPHOCYTES

No significant differences in the relative proportion of CD4+ or CD8+ subsets were observed in CD3+ cells from UC or FAP patients with ileoanal pouches without signs of inflammation or in the ileal mucosa proximal to the pouch (table 2). In contrast, CD3+ T cells in pouchitis were 45.4% CD4+ (range 36.9–71.1%), whereas in uninfamed pouch mucosa only 30.2% (9.1–45.3%) were CD4+ (see table 2). Figure 1 shows a representative immunofluorescence profile. The CD4:CD8 ratio was significantly increased in pouchitis (1.3 (0.7–2.7) compared with 0.6 (0.1–1.0)) in uninfamed pouches or in ileal mucosa (0.4 (0.3–0.4)) ($p=0.012$; see fig 2 and table 2). Analysis of peripheral blood CD3+ cells revealed no significant differences in CD4:CD8 ratio in patients with and without pouchitis. The CD4:CD8 ratio in peripheral blood of patients with UC with active pouchitis was 0.9 (range 0.7–1.1), and in patients with UC without the pouchitis the CD4:CD8 ratio was 0.8 (0.7–1.2).

Figure 2 Intraindividual comparison of CD4:CD8 ratio of mucosal T cells isolated from IAP and the adjacent ileum. Bars represent the median of all patients; lines connect the intraindividual values of the CD4:CD8 ratio.

Table 2 T cell subsets and degree of activation in inflamed and non-inflamed pouch and ileal mucosa

	UC with pouchitis		UC without pouchitis		FAP without pouchitis	
	Pouch (n=8)	Ileum (n=5)	Pouch (n=10)	Ileum (n=7)	Pouch (n=9)	Ileum (n=3)
CD3+/CD4+	45.4 (36.9–71.1)	23.4 (18.0–25.9)	30.2 (9.1–45.3)	20.5 (20.1–39.9)	35.3 (30.9–66.7)	44.2 (24.8–52.9)
CD3+/CD8+	42.5 (25.4–57.4)	62.5 (59.3–72.6)	54.8 (35.6–82.0)	72.0 (46.9–80.8)	48.9 (22.7–57.1)	42.9 (30.6–51.7)
CD4:CD8 ratio	1.3 (0.7–2.7)*	0.4 (0.3–0.4)	0.6 (0.1–1.0)	0.3 (0.2–0.8)	0.9 (0.5–2.9)	1.0 (0.5–1.7)
CD4+/CD25+	16.0 (8.6–20.8)†	7.7 (4.0–12.0)	11.9 (3.2–20.4)	3.3 (1.1–8.7)	9.3 (2.0–17.8)	3.1 (2.8–7.0)
CD8+/CD25+	8.6 (2.2–16.5)	7.1 (2.4–12.0)	7.4 (1.1–48.7)	2.0 (1.8–3.8)	4.5 (0.0–14.2)	3.5 (2.4–4.6)
CD4+/HLA-DR+	10.7 (6.8–34.6)	7.9 (3.6–10.3)	12.3 (1.2–28.9)	8.2 (4.8–12.4)	17.9 (6.8–33.6)	10.5 (6.2–22.5)
CD8+/HLA-DR+	19.9 (11.2–31.5)‡	2.1 (1.6–5.7)	11.3 (3.6–38.5)	5.1 (2.1–16.2)	12.5 (1.9–42.1)	4.3 (2.0–34.4)

* $p=0.012$, † $p=0.036$, ‡ $p=0.05$.

Figure 3 CD4:CD8 ratio and crypt lengths in individual patients during and after pouchitis. Lines connect the intraindividual values for the CD4:CD8 ratio and crypt depth measured in biopsy specimens obtained from the same patient during acute pouchitis and after treatment with normalisation of clinical and endoscopic signs of inflammation.

Figure 4 IFN- γ production in LPMNCs of patients with UC with and without pouchitis.

EXPRESSION OF ACTIVATION AND DIFFERENTIATION MARKERS ON MUCOSAL T CELL SUBSETS IN POUCHITIS AND NON-INFLAMED POUCHES

Using three colour flow cytometry, the relative numbers of CD4+ and CD8+ cells which are CD25+, CD45R0+, and HLA-DR+ were determined in patients with and without

pouchitis. In pouchitis the relative numbers of CD4+/CD25+, and CD8+/HLA-DR+ were increased compared with those in patients with UC without pouchitis (table 2). However, the numbers of CD4+/CD25+, CD4+/HLA-DR+, CD8+/CD25+, and CD8+/HLA-DR+ T cells in the non-inflamed mucosa of UC and FAP patients were comparable. In additional studies, it was shown that approximately 95% of the CD3+ lymphocytes were CD45R0+ without any differences in all groups (data not shown).

INTRAINDIVIDUAL COMPARISON OF MUCOSAL T CELL SUBSETS IN PATIENTS WITH AND WITHOUT POUCHITIS

As flow cytometry revealed an increased CD4:CD8 ratio of mucosal T cells in pouchitis, we investigated the effect of medical treatment using metronidazole on distribution of CD3+ cells in four patients with acute pouchitis. After a median follow up of three months a significantly decreased CD4:CD8 ratio in each patient was observed after improvement of clinical symptoms and endoscopic/histological signs of pouchitis (fig 3). Due to technical reasons, analysis of the CD4:CD8 ratio during follow up examinations was not possible in four of eight patients who were studied. Data for these eight patients are summarised in table 2.

PRODUCTION OF IFN- γ

Under basal conditions, the number of IFN- γ producing cells in LPMNCs was significantly higher ($p=0.028$) in pouchitis (mean 6.5 (0.7) per 10^5 cells) compared with that in pouches without inflammation (0.7 (0.3) per 10^5 cells). Furthermore, after stimulation using the mitogen PHA a significant increase in IFN- γ producing cells in both conditions was observed. However, the differences between pouchitis and uninflamed pouches remained (fig 4). Interestingly, no differences between the number of IFN- γ positive cells derived from ileal biopsy specimens in patients with or without inflamed pouches were detected.

MORPHOMETRY

Pouch mucosal biopsy samples were assessed by three dimensional measurement of micro-dissected specimens. There was a notable diversity in morphometric data in single biopsy specimens from patients with pouchitis compared with uninflamed pouch mucosa. Median villous length was 376 μ m, and median villous surface area was 0.31 mm^2 in pouchitis, compared with a villous height of 393 μ m, and median villous surface area of 0.26 mm^2 in uninflamed pouch mucosa (table 3). Crypt depth was significantly increased in patients with UC with pouchitis (352 μ m) compared with that in patients with UC without inflamed pouches (239 μ m) or FAP patients (270 μ m) ($p=0.012$). Intraindividual comparison of crypt depths in patients with pouchitis ($n=6$) indicated a decrease in crypt depth from 360 μ m (median) to 250 μ m after improvement of pouchitis (fig 3). Statistical analysis revealed a correlation between crypt depth and the

Table 3 Morphometric data for inflamed and non-inflamed pouch and ileal mucosa

	UC with pouchitis		UC without pouchitis		FAP without pouchitis	
	Pouch (n=8)	Ileum (n=5)	Pouch (n=10)	Ileum (n=7)	Pouch (n=9)	Ileum (n=3)
Villous length (μm)	376 (21–637)	595 (527–720)	393 (293–511)	533 (304–659)	406 (334–463)	490 (332–569)
Crypt depth (μm)	352 (291–492)	181 (129–418)	239 (141–422)	171 (136–215)	270 (241–417)	196 (159–238)
Villous surface area (mm^2)	0.31 (0.1–0.62)	0.41 (0.37–0.74)	0.26 (0.19–0.30)	0.28 (0.14–0.42)	0.21 (0.17–0.38)	0.32 (0.27–0.41)

Figure 5 Correlation between the percentage of mucosal CD4+ T cells in pouch mucosa and the crypt depth in biopsy specimens from patients with UC and ileoanal pouches.

relative number of mucosal CD4+ cells ($r=0.57$; $p=0.011$) in all patients with UC (fig 5).

Discussion

Current concepts of the pathogenesis of pouchitis postulate that pouchitis in patients with IAP after proctocolectomy might represent a recurrence of the underlying inflammatory disease. In favour of this hypothesis are several clinical findings such as the presence of extraintestinal manifestations in pouchitis and the association of pouchitis with a history of UC.²⁷ In addition, as in active UC, immunohistochemical studies have shown an increase in IgG containing plasma cells and RFD9+ macrophages in pouchitis.^{8, 27–29} In order to characterise mucosal lymphocytes and cytokine expression in this condition, we isolated lymphocytes and determined the phenotype and state of activation in ileoanal pouches from patients with and without pouchitis. The first substantial finding in this study was the significant increase in the proportion of CD4+ mucosal lymphocytes in pouchitis compared with mononuclear cells from uninfamed pouches and normal ileal mucosa. Furthermore, the expression of activation markers was also significantly increased on CD4+ and CD8+ T cells in pouchitis. Expression of CD25 on CD4+ cells was more pronounced than on CD8+ T cells; this is a well known phenomenon of T cell activation.³⁰ A similar increased state of activation of T cells in active UC was described by us and other groups indicating parallels in the pathogenesis of UC and pouchitis.³¹ However, to our knowledge an increased CD4:CD8 ratio of mucosal lymphocytes as observed in our study has not been described in active or chronic UC. In this context, it is important to note that the incidence

of chronic or severe pouchitis is increased in patients with UC with backwash ileitis.³² Therefore, analysis of mucosal T cell subsets in backwash ileitis is of major interest. Since pouchitis in FAP patients with ileoanal pouches is very rare, we were able to analyse mucosal lymphocytes in only one FAP patient with macroscopic and histological signs of inflammation. In this patient the CD4:CD8 ratio was 2.1 and the degree of activation of CD4+ and CD8+ T cells was similar to pouchitis in UC (data not shown).

A possible limitation of this study is that our mucosal mononuclear cell preparation represents a mixture of intraepithelial and lamina propria lymphocytes. As the majority of intraepithelial lymphocytes are CD8+ cells, it is possible that the observed increase in the CD4:CD8 ratio in pouchitis reflects a simple reduction of intraepithelial lymphocyte density in inflammation compared with non-inflamed pouches. However, this is unlikely as immunohistochemical characterisation of intraepithelial lymphocyte density revealed no differences in pouches with and without inflammation (analysis done by I Müller-Molaian in blinded manner, data not shown).

The second important result in this study was the significantly increased number of IFN- γ producing cells in biopsy specimens derived from inflamed pouches. IFN- γ , a cytokine secreted by activated T cells and natural killer (NK) cells promotes inflammation—for example, by activating macrophages and up regulating the expression of cell adhesion molecules.³³ As MHC antigen expression is strongly induced by IFN- γ , the increased number IFN- γ producing cells might provide an explanation for the finding that epithelial cells derived from inflamed mucosa of IAP patients expressed higher levels of MHC class II antigens compared with uninfamed pouches. Furthermore, the increased IFN- γ production might be responsible for the tissue damage observed in pouchitis, as IFN- γ is able to sustain cytotoxic reactions.³⁴

Although the maintenance of the structure of the small intestinal mucosa may be disturbed by various types of stress, the response of the mucosa is rather uniform and restricted to three distinct patterns: hypertrophy, atrophy, and mucosal transformation of the hyper-regenerative type with villous atrophy and crypt hyperplasia.³⁵ Mild villous atrophy is a common histological feature in ileoanal pouches. Using morphometric analysis we showed a clear association between crypt depth and the relative number of CD4+ mucosal T lymphocytes in acute and chronic inflammation of the pouch. In addition, activation of CD4+ and CD8+ T cells in acute inflammation was

observed in most cases of crypt hyperplasia in the pouch mucosa. Other investigators did not find such a correlation.³⁶ However, in these studies quantitative measurement of mucosal architecture was done using microscopic sections, a method which is less accurate than three dimensional morphometry.^{26, 37} As evidenced in previous studies on mucosal transformation in the course of HIV enteropathy, three dimensional morphometry is a suitable technique to evaluate discrete changes in villous length and crypt depth. Using morphometric analysis, we were able to describe a hyporegenerative atrophy of the small intestine in HIV infected patients.³⁹

Several experimental and clinical findings clearly indicate that activated T cells interact with intestinal epithelial cells and influence viability and proliferation of these cells. For example, incubation of the colonic carcinoma derived cell line HT29 with supernatants of anti-CD3 stimulated T cells or with tumour necrosis factor α resulted in a decreased viability as measured by vital propidium iodide staining and induction of apoptosis, thus confirming a functional inter-relation between mucosal structure and the mucosal immune system.^{40, 41} Intestinal T cell activation is increased in coeliac disease and intestinal infections.^{42, 43} In addition, it has been shown in vitro that activation of T cells resulted in mucosal destruction of fetal small intestinal explants with complete villous atrophy, aphthous lesions, and focal ulcerations. The addition of dexamethasone prevented mucosal destruction, but explants developed a sprue-like transformation of the small intestinal mucosa with villous atrophy and crypt hyperplasia.^{20, 21} In contrast, the depletion or functional impairment of activated lamina propria T cells in HIV infected patients is accompanied by a hyporegenerative adaptation (atrophy).³⁸ From these clinical and experimental findings and from the results of the present study it can be hypothesised that a correlation exists between the relative number of activated CD4+ mucosal T lymphocytes and mucosal structure in the small intestine: loss of these CD4+ T cells may result in hypotrophy as seen in HIV infection—that is, a physiological state of activation of CD4+ T cells in the lamina propria may be necessary for the maintenance of the normal mucosal structure. An increased “pathological” state of activation of CD4+ lymphocytes, on the other hand, may correlate with mucosal transformation of the hyper-regenerative type as seen in pouchitis. The high activation status of mucosal CD4+ T cells and elevated number of IFN- γ producing cells might thus lead to mucosal destruction with focal ulcerations, aphthous lesions, and crypt hyperplasia as seen in pouchitis after ileo-anal pouch anastomosis.

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paper is dedicated to Prof. Dr Ernst Otto Riecken, Berlin, Germany, on the occasion of his 65th birthday.

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