Leucocyte typing, cytokine expression, and epithelial turnover in the ileal pouch in patients with ulcerative colitis and familial adenomatous polyposis


Abstract

Background/Aims—Conventional histopathology, leucocyte typing, cytokine mRNA expression, and crypt cell turnover were compared in ileal pouch biopsy specimens from patients with ulcerative colitis (UC) and familial adenomatous polyposis (FAP).

Methods—Biopsy specimens were taken from 17 patients with UC and seven with FAP at a median interval of 19 months (range 2–120) after ileostomy closure. All contained both epithelium and lamina propria. Cryostat sections were stained for lymphocyte subtypes (CD3, CD4, CD8), macrophages (CD68), common leucocyte antigen (CD45), and Ki-67, using a three stage immunoperoxidase reaction. Cytokine mRNA expression for interleukins 2 and 6, tumour necrosis factor α, and interferon γ was studied using an in situ hybridisation technique.

Results—Lymphocyte subtype and macrophage populations in epithelium and lamina propria were similar in UC and FAP. The labelling index (Ki-67) was significantly increased in biopsy specimens from patients with UC (UC median = 43.3 (interquartile range (IQR) 38.9–48.2) v FAP 34.9 (29.9–35.2), p<0.005). There was little or no epithelial mRNA expression for any cytokine in any of the specimens. Lamina propria mRNA expression for interleukin 2 was significantly increased in UC (UC median (IQR) 10.7 (5.4–14.2) cells per unit area v FAP 2.8 (1.5–6.6) p<0.005) but not for tumour necrosis factor α, interleukin 6, and interferon γ.

Conclusions—While static morphological assessment (leucocyte type, conventional histopathological examination) was similar, tests of cell function (mRNA expression and labelling index) were different in ileal pouches in patients with UC compared with FAP. The study also showed that mRNA expression occurred almost entirely in the lamina propria.

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Keywords: leucocyte typing, cytokine expression, ulcerative colitis, familial adenomatous polyposis, ileal pouch, epithelial turnover.

The ileal mucosa undergoes morphological changes after ileoanal reservoir construction. These include villous atrophy and crypt hyperplasia, which occur in almost all patients. The enterocytes may express colonic antigens and produce colonic type mucins. Leucocyte typing in the terminal ileal mucosa before and after reservoir construction has shown a change to a pattern similar to that of the inflamed rectum in ulcerative colitis. The clinical condition of pouchitis occurs commonly in UC, but has seldom been identified where the operation has been performed for familial adenomatous polyposis (FAP). There are therefore obvious morphological and clinical differences in the pouch mucosa of the two diseases.

Disease specific changes at a cellular level have not been studied in detail. They are probably important because the pouch in colitic patients might offer a model of the evolution of inflammatory bowel disease in humans. The pouch in FAP should provide a comparison as this is a neoplastic disorder.

The study aimed to identify possible differences in the ileoanal pouch mucosa in patients with UC and FAP with regard to epithelial cell turnover and the inflammatory response. In addition the study sought to determine the localisation of any response whether in the epithelium or lamina propria.

Five cellular and molecular variables were examined relating to inflammation and immune response in ileal mucosa from patients with UC and FAP. Staining by haematoxylin and eosin was used for baseline morphological comparison based on a previously described scoring system. Immunocytochemical techniques were used to determine the presence and distribution of helper and cytotoxic lymphocytes and macrophages and mucosal cell turnover was assessed by Ki-67 distribution. The expression of mRNA for interleukin 2 and 6 and tumour necrosis factor α and interferon γ was determined by in situ hybridisation.

Methods

The study required the presence of both epithelium and lamina propria in the same biopsy specimen. Accordingly patients with ulceration preventing assessment of the epithelium were excluded. The study comprised biopsy specimens from 17 patients with UC (mean age 39 years range 20–63, 15 males)
and seven with FAP (mean age 35 years range 21–44, 5 males). Samples were taken at a mean interval of 19 months (range 2–120) after closure of the temporary ileostomy.

The study was approved by the ethical committee of the City and Hackney Health Authority, West Smithfield, London.

One biopsy specimen was taken from each patient. This was snap frozen and stored in liquid nitrogen until sectioning. Cryostat sections (5 μm) were taken and stained with haematoxylin and eosin for morphological examination. Immunoperoxidase staining as previously described was then carried out for the following analyses: CD45 (pan leukocyte antigen), CD68 (macrophages), CD3 (T lymphocytes), CD4 (helper/inducer T cells), CD8 (suppressor/cytotoxic T cells), Ki-67 (proliferating crypt cells). Table I shows the antibodies and respective dilutions used. Briefly, after fixation in aceton at 4°C for five minutes, the sections were incubated with the primary antibodies for 45 minutes. They were then incubated in rabbit antirabbit immunoglobulin, then with streptavidin-biotin complex at 1:100 dilution for 30 minutes. The peroxidase reaction was developed with diaminobenzidine (0-5 mg/ml). Sections were washed thoroughly with TRIS buffered saline between each of the above steps. Mayer’s haematoxylin was used to counterstain and sections were dehydrated in alcohol and mounted under coverslips in DPX (BDH).

Cytokine mRNA was detected by the use of a cocktail of three DNA oligonucleotide probes that were prepared in house, for the cytokines tumour necrosis factor α, interferon γ, interleukin 2 and 6 using a previously described method. The specificity of the probes was confirmed by northern blotting. Briefly, after fixation in paraformaldehyde in phosphate buffered saline (PBS), sections were washed in sucrose in PBS. They were treated with Triton-x-100, proteinase K, paraformaldehyde, and tritethanolamine containing acetic anhydride. Sections were then placed in a mixture of saline-sodium citrate buffer (SSC), formamide, and Denhardt’s solution followed by hybridisation overnight with 2 ng of labelled probe in a solution containing formamide, denatured sheared salmon sperm DNA, and dextran sulphate. After hybridisation sections were washed in SSC and formamide, dehydrated in alcohol, and air dried. Sections were then dipped in Ilford K5 photographic gel emulsion, dried in a dark room, and incubated at 4°C in a light proof box containing silica gel for five days. Slides were developed in Dektol solution (Kodak), washed, fixed, and counterstained with Mayer’s haematoxylin.

Controls

For the immunoperoxidase staining method, negative controls for each antibody were made by replacing the specific antibody with its diluent (TBS). An additional negative control involved an irrelevant murine antibody MAC 181. In the method used for the detection of cytokine mRNA, RNase-free DNase (no effect on hybridisation), and DNase-free RNase (no hybridisation), as well as oligonucleotides containing sequences complementary to the cytokine probes (sense probes) were used as negative controls. An oligo-Dt probe for the detection of total mRNA was used as a positive control.

Counting protocol

Haematoxylin and eosin morphology was recorded blind by a single observer (ICT) using the St Mark’s scoring system. For leukocyte typing in the epithelium, the positively stained cells were expressed as a percentage of all epithelial cells. These included both enterocytes and intraepithelial lymphocytes. In the lamina propria cells were counted per unit area (grid; 0-0025 mm²). At least 500 epithelial cells and three consecutive areas in the lamina propria (within and below the villi going to deeper lamina propria) were counted for each antibody. Counts were expressed as positive cells per 100 nuclei in both the surface epithelium and lamina propria.

For cytokine mRNA determination, it was not possible to express lamina propria counts per 100 nuclei owing to the destruction of some nucleated cells during processing. Despite counterstaining, some of the cell nuclei were not visible as discrete nuclei. Counts were therefore expressed as positive cells per unit area (grid; 0-0025 mm²). At least three grids were counted for each specimen and the results averaged.

The antibody Ki-67 identifies an epitope on dividing cells and its location in the epithelium is an indirect measure of crypt cell turnover. The number of Ki-67 positive and

<table>
<thead>
<tr>
<th>Antibody</th>
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<th>Dilution</th>
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<tbody>
<tr>
<td>CD45</td>
<td>Serotec</td>
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<td>Common leucocyte antigen</td>
</tr>
<tr>
<td>CD68</td>
<td>Serotec</td>
<td>1:20</td>
<td>Macrophage</td>
</tr>
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<td>CD3</td>
<td>Dako</td>
<td>1:80</td>
<td>All T lymphocytes</td>
</tr>
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<td>CD4</td>
<td>Dako</td>
<td>1:20</td>
<td>Helper/inducer lymphocytes</td>
</tr>
<tr>
<td>CD8</td>
<td>Dako</td>
<td>1:20</td>
<td>Suppressor/cytotoxic T cells</td>
</tr>
<tr>
<td>Ki-67</td>
<td>Dako</td>
<td>1:20</td>
<td>Proliferating cells</td>
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<table>
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<th>FAP</th>
<th>Total processed</th>
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<td>17</td>
<td>7</td>
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**Histological score**

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</tr>
<tr>
<td>Macrophage (CD68)</td>
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<td>9</td>
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<tr>
<td>Lymphocyte subtypes (CD3, CD4, CD8)</td>
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<td>17</td>
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<tr>
<td>IE</td>
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**In situ hybridisation:**

<table>
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<th>Tumour necrosis factor α</th>
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<tbody>
<tr>
<td>IE</td>
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</tr>
<tr>
<td>LP</td>
<td>8</td>
</tr>
<tr>
<td>Interferon γ</td>
<td>8</td>
</tr>
<tr>
<td>Interleukin 2</td>
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<tr>
<td>Interleukin 6</td>
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</tr>
<tr>
<td>Ki-67</td>
<td>17</td>
</tr>
</tbody>
</table>

IE = intraepithelial, LP = lamina propria.
negative epithelial cells within a minimum of three well oriented crypts cut along their entire length in each section were counted and averaged. In sections with few or no complete crypts, at least six halves of different crypts (crypt columns) were counted. The labelling index was given by the ratio of Ki-67 positive to the total number of epithelial cells expressed as a percentage.

**Counting accuracy**

A random selection of sections was recounted by a second observer and the results were found to be within 10 per cent of those presented in this paper.

**Statistical analysis**

All data were treated as non-parametric and presented as median and interquartile range (IQR). Significance was tested using the Mann-Whitney U test with p<0.05 accepted as significant.

**Results**

Table II shows the number of biopsy specimens examined for each antibody. Haematoxylin and eosin scoring was available for all samples. Some sections were damaged during processing thereby reducing the numbers available for analysis.

There was no statistically significant difference between UC and FAP in the histological scores for acute cellular infiltration (median (IQR) 1 (0–1) UC v 1 (0–1) FAP; p=0.9), ulceration (0 (0–0) UC v 0 (0–0) FAP; p=1.0), chronic cellular infiltration (2 (1–2) UC v 1 (1–2) FAP; p=0.3) or villous atrophy (1 (1–2) UC v 1 (0–2) FAP; p=0.7).

The distribution of CD45, CD68, CD3, CD4, and CD8 positive cells was similar within both epithelium and lamina propria in both disease groups (Table III).

There was little or no epithelial signal for cytokine mRNA expression in any patient in either disease group (0–1.5%). In the lamina propria, however, cytokine mRNA expression was strongly present in cells and was significantly greater for interleukin 2 in UC than in FAP (UC median (IQR) 10·7 (5·4–14·2) v FAP 2·8 (1·5–6·6) p<0.05). Other mRNA cytokine expression was not significantly different (Table IV).

There was no hybridisation with the sense probes, showing that binding of the antisense probes was specific. There was an absence of hybridisation in sections treated with RNase showing that the hybridisation was with mRNA.

Labelling index as estimated by Ki-67 was significantly greater in UC than FAP (Table V).

However, for the whole group and the UC subgroup there was no correlation between interleukin 2 lamina propria counts and labelling index (n=11, r=0·77, r²=0·01, and p=0.75, r²=0.02, respectively).

**Discussion**

FAP and UC are two different pathological processes. The ileal pouch undergoes morphological changes common to both, particularly...
inflamed mucosa would have prevented the study because the resulting distortion, including ulceration, would have made morphological study of the epithelial layer impossible. In addition, the ulceration itself would have changed the inflammatory response deep to it. By selecting UC and FAP pouch mucosa microscopically similar on haematoxylin and eosin staining, we explored the hypothesis that there might be differences in cell type and cytokine expression, which might be related to the intrinsic disease. It was also felt that such a selection might minimise any distortion of the mucosa resulting from oedema.

Although the in situ hybridisation sections were counterstained for nucleated cells, it was impossible to count these as the cells in the lamina propria are damaged by the technique. Counting positively stained cells per unit area was the only feasible means of quantification. The similarity of haematoxylin and eosin histological scores in the UC and FAP sections particularly of acute inflammation is evidence that oedema was unlikely to be a major source of error.

Ethical permission allowed only one biopsy specimen per patient. Suitably stained sections were not obtained from all of these although most were of adequate quality for counting as shown in Table I. In some parts of the study therefore, the numbers were small and further study is clearly necessary, especially in the case of mRNA cytokine expression, where a trend between the two groups was seen with interleukin 6. The lymphocyte subtype ratios were similar to those reported by de Silva confirming the quality of counting. The summation of lymphocyte subtypes within the lamina propria was always below total nucleated cell counts and counts for CD4 and CD8 summed, approached those of CD3. CD68 and CD3 did not exceed those for CD45.

Another possible source of inaccuracy is due to the total depth of the section taken within the biopsy specimen. Each was 5 μm thick but in a consecutive series of 40 sections, /200 μm of tissue was sampled. Each component of the study was done sequentially,
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minimising the morphological variance that might have resulted by heterogeneity within the sample. All these sources of inaccuracy are inherent in the methodology of cytochemistry.

Interleukin 2 is mainly produced by CD4 positive cells. Its actions include activation of T cells, macrophages, and the growth and differentiation of B cells. The significantly increased interleukin 2 mRNA expression in the lamina propria of pouch mucosa in UC suggests T cell activation in this mucosa. This supports an immunological basis for the development of the more severe inflammatory changes seen in pouchitis.

The difference between expression of tumour necrosis factor α and other cytokines in ileal pouches has been observed by others. Gionchetti et al measured interleukin 1b, interleukin 6, interleukin 8, and tumour necrosis factor α concentrations in biopsy specimens from ileal pouches and were unable to detect any tumour necrosis factor α activity in any specimen while showing increased concentrations of the others.

A causal relation has been shown between crypt cell proliferation and T cell activation in cultured fetal colon. In this study both these processes were upregulated in UC, and in both disease processes the expression of mRNA for interleukin 2 was confined to the lamina propria. As no correlation was found, however, between labelling index and interleukin 2 counts in the lamina propria, crypt cell proliferation does not seem to be directly influenced by actions of this particular cytokine.

The results of the study show that static morphological assessment of inflammation in the ileal pouches is unlikely to give information on the biological processes involved. In contrast, study of the dynamic aspects of the immune process and inflammatory response can do so.

In the former case, no difference between UC and FAP was found in lymphocyte subtype proportions and numbers of macrophage and general leucocyte antigen positive cells. In the latter case, the significant differences in numbers of Ki-67 positive cells and mRNA interleukin 2 expression seen in UC show that further work should be aimed at the study of dynamic changes and their evolution.

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