IL-12 and T-lymphocyte dependant mucosal immune respons

Camoglio, L.

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Chapter 2

Altered expression of interferon-γ and interleukin-4 in inflammatory bowel disease
Altered Expression of Interferon-γ and Interleukin-4 in Inflammatory Bowel Disease

*†Luisa Camoglio, *Anje A. Te Velde, †Albert J. Tigges, †Pranab K. Das, and *Sander J. H. Van Deventer

*Laboratory of Experimental Internal Medicine; and †Research Laboratory of Pathology (Dermato-Immune Pathology), Academic Medical Centre, University of Amsterdam, The Netherlands

Summary: Experimental data indicate that mucosal CD4+ T cells play an important role in the pathogenesis of inflammatory bowel disease (IBD). Based on the pattern of cytokine production, CD4+ T cells may be distinguished into two different phenotypes. Th1 responses are characterized by secretion of interleukin (IL)-2, tumor necrosis factor (TNF)-α, lymphotoxin, and interferon (IFN)-γ and are associated with delayed-type hypersensitivity reactions, whereas Th2 responses, which are characterized by secretion of IL-4, IL-5, and IL-10, have been associated with humoral immune responses and allergy. To assess the number of IFN-α and IL-4 positive cells in IBD and normal intestinal specimens, frozen sections from intestinal specimens from 10 Crohn’s disease (CD), 8 ulcerative colitis (UC), and 8 healthy controls were examined by immunohistochemistry. Monoclonal antibodies for CD3, CD8, IFN-γ, and IL-4 were used. T-lymphocyte infiltration and cytokine expression by epithelial, lamina propria, and submucosal cells were scored on a four-point scale by two independent observers who were blinded for the clinical data. One-way analysis of variance (ANOVA) testing was used for statistical analysis. In intestinal specimens from IBD patients, the number of CD3+ cells was found increased in the lamina propria and, within the submucosa, this increase was significant (p < 0.001). In CD the number of lamina propria IFN-γ positive cells was significantly increased as compared with controls (p < 0.002). In UC the number of both IFN-γ and IL-4 producing cells in the lamina propria was not significantly increased as compared with controls. The present results confirm the existence of a Th1-biased pattern production in CD but not in UC. Key Words: Inflammatory bowel disease—Crohn’s disease—Ulcerative colitis—Cytokines—Interferon-α—Interleukin-4—Immunohistochemistry

INTRODUCTION

Antigen-dependent T-cell stimulation results in differentiation into various phenotypes that are characterized by specific cytokine profiles. Th1 cells produce interleukin-2 (IL-2), interferon-γ (IFN-γ), and tumor necrosis factor-α (TNF-α), and mediate cellular immune responses, whereas Th2 cells, which produce IL-4, IL-5, IL-10, and IL-13, are implicated in humoral responses and allergy.

T lymphocytes have an important role in the pathogenesis of intestinal inflammation, and increased production of IFN-γ is a hallmark of Crohn’s disease (CD) (1). Recently, two studies have demonstrated increased mucosal production of IL-12, the major inducer of IFN-γ, in active CD (2,3). In contrast, ulcerative colitis (UC) is characterized by increased production of IL-5 (4). These data suggest that the immunological mechanisms that underlie CD and UC markedly differ. Th1-type cytokine production is generally increased in CD, whereas a Th2 type pattern is found in UC. However, this scheme is complicated by reports that in early CD lesions, mucosal IL-4 messenger ribonucleic acid (mRNA) dominated and IFN-γ mRNA decreased (5). Moreover, increased mucosal production of IL-10 was found increased in both active CD and UC (6).

Most studies on mucosal cytokine production have used methods that measure total mucosal cytokine production (enzyme-linked immunosorbent assay [ELISA], reverse transcribed polymerase chain reaction [RT-PCR]), or cytokine production by isolated lamina propria mononuclear cells. These methods do not allow for analysis of the distribution of cytokine producing cells within the mucosal layers.

In the present study we have analysed the mucosal
distribution of cells producing IFN-γ and IL-4 in resection specimens obtained from patients with CD, UC, and from noninflammatory controls. Here we confirm the presence of increased numbers of IFN-γ-positive lymphocytes in CD, and report that these cells are mainly located within the lamina propria compartment.

**MATERIALS AND METHODS**

**Patients**

Resection specimens were obtained from 10 patients with CD (4 male, 6 female) who underwent bowel resection because of medical treatment failure (n = 4), extensive inflammatory infiltrate (n = 5), or fistula formation (n = 1). The mean age of this group was 32 years (range, 17-54 years) and the disease duration ranged from 1-19 years (mean, 6.2 years). Four patients had ileal disease, five had colonic localization, and in one patient both colon and ileum were involved. Seven patients used corticosteroids.

Eight patients (three male, five female) with UC. mean age 32.8 years (range, 18-54 years), underwent total colectomy. Their disease duration was 5.4 years (range, 0.2-15 years). Five patients had shown insufficient reaction to medical treatment and seven patients were on corticosteroid treatment.

The control group consisted of eight normal specimens obtained from patients who had a bowel resection for various reasons (two carcinoma, two diverticulitis, one polyposis coli syndrome, one ischemia, one perforation, and one adenomatous polyp).

Specimens were snap-frozen in liquid nitrogen and stored in -70°C until analysis. Sections were cut and tissues stained with hematoxylin and eosin for histologic analysis.

**Immunohistochemistry**

The single stain protocol was as follows. Serial tissue sections (6 μm) were cut on a cryostat, mounted on gelatine-coated glass slides, and fixed in ice-cold acetone. Staining was performed according to a three-step immunoperoxidase method.

After a brief wash in phosphate buffered saline (PBS), sections were incubated for 30 minutes in 10% normal goat serum (Dako A/S, Glostrup, Denmark) and then incubated with the first antibody diluted in PBS/1% bovine serum albumin (Sigma, St. Louis, MO, U.S.A.) 1 hour at room temperature (monoclonal mouse antihuman-IFN-γ and -IL-4 antibodies, Genzyme, Cambridge, MA, U.S.A.). Endogenous peroxidase activity was quenched by placing the slides in a solution of methanol/1% H2O2 for 20 minutes. Following a further wash in PBS, sections were incubated with biotinylated rabbit antimouse antibody (Dako) diluted 1:200 in PBS/10% AB serum for 30 minutes (CLB, Amsterdam, The Netherlands) and then with streptavidin complex (Dako) for 30 minutes. Slides were developed using hydrogen peroxide (H2O2) and 3,3-aminophenol (Sigma) and N,N-dimethylformamide (Sigma) in acetate buffer. Following counter-staining with hematoxylin, sections were coated with glycerol and a cover slip was applied. In control sections, the primary antibody was omitted. In double-staining experiments, a mouse antihuman-IL-4 antibody was incubated overnight at 4°C, and after a wash in PBS a rabbit antihuman-CD3 was incubated for 1 hour at room temperature. Endogenous peroxidase activity was quenched by placing the slides in a solution of methanol/1% H2O2 for 20 minutes. Following a further wash in PBS, sections were incubated with alkaline phosphatase-conjugated swine-antirabbit (Dako) and biotin-conjugated goat-antimouse (Southern Biotechnology Associates, Birmingham, AL, U.S.A.) and diluted in TBS/10% AB serum were incubated together for 30 minutes and then with streptavidin complex (Dako) for 30 minutes. Alkaline phosphatase activity (blue staining) was detected using Fast Blue (Sigma) and naphthol-AS-MX-phosphate (Sigma), to which levamisole (Sigma) was added; peroxidase activity (red activity) was detected using 3,3-aminophenol carbazole and N,N-dimethylformamide.

Two different isotype antibodies, mouse antihuman-CD3, IgG1 (incubated overnight at 4°C), and mouse antihuman IFN-γ, IgG2a (incubated 1 hour at room temperature), were used as functional markers for T cells and IFN-γ and alkaline phosphatase-conjugated goat-antimouse-IgG1 (Southern Biotechnology Associates) and peroxidase-conjugated goat-antimouse-IgG2a (Southern Biotechnology Associates) were used as secondary antibodies. Alkaline phosphatase and peroxidase activities were detected as previous described.

**Microscopic Analysis**

T-cell infiltration and cytokine expression within the epithelial, lamina propria, and submucosal layers were scored on a four-point scale by two independent observers who were blinded for clinical data. A score of 0 represented minimal infiltration, while a score of 4 represented infiltration by numerous inflammatory cells. Individual readings were identical or differed by only one
point. Minimal differences between the observers were resolved by mutual agreement.

**Statistical Analysis**

Differences between the three patient groups were tested by one-way analysis of variance using SPSS statistics for Windows (SPSS, Inc., Chicago, IL, U.S.A.). A two-sided p < 0.05 was considered significant.

**RESULTS**

Histological analysis showed active inflammation in all 10 CD patients and the presence of granulomas in 8 specimens (data not shown). Five UC patients had active colitis and three had mild inflammation. Table 1 summarizes the clinical data of both IBD populations. The three groups were well matched for age, sex ratio, duration of disease, and localization of disease. The corticosteroid use in CD and UC patients was equally distributed.

Monoclonal antibodies specific for CD3, CD8, IFN-γ, and IL-4 were used in single-staining (Fig. 1) and CD3 in combination with either IFN-γ or IL-4 in double-staining. T-cell infiltration was scored on epithelial, lamina propria, and submucosal compartments (Table 2). The number of intraepithelial CD3+ T cells was slightly (nonsignificantly) increased in CD as compared with UC and controls. The number of CD3+ T cells in lamina propria was somewhat increased in both diseases compared with controls, but the most striking difference was observed in the submucosa of CD specimens that contained an increased number of CD3+ T cells (p < 0.001). The pattern of epithelial and lamina propria CD8+ cells revealed no significant differences in both diseases, as compared with controls with the only exception of an increase of CD8+ T cells in the submucosal compartment in CD patients (p = 0.002). By analyzing single-stained serial sections, it was shown that the majority of CD3+ T cells were CD4+ T cells.

The number of lamina propria IFN-γ+ cells was significantly greater in CD compared with controls (p < 0.002) (Fig. 3), and the morphology of lamina propria IFN-γ+ cells was typical of macrophages and lymphocytes (Fig. 1). In UC, the pattern of IFN-γ+ cells showed no difference between the patient material and normal tissue, whereas the number of lamina propria IL-4+ cells was increased, but not significantly (Figs. 2 and 3). In CD, the number of lamina propria and submucosal IL-4+ cells was slightly and not significantly increased as compared with normal specimens (Fig. 3).

**DISCUSSION**

We report that the number of lamina propria CD3+ (T lymphocytes) cells in both CD and UC was increased. The number of submucosal CD3+ cells was increased in both diseases as compared with normal specimens (p = 0.001).

IFN-γ+ cells were more often present in the lamina propria of CD specimens as compared with UC and with controls (p < 0.002). No differences were seen in the epithelial and submucosal compartments between the three groups. In UC, the number of IL-4+ cells was only slightly and nonsignificantly increased as compared with normal controls, and the pattern of IFN-γ+ cells within the three examined compartments did not differ from controls.

These results are in accordance with several previous studies that reported an increase of the number of CD4+ T cells in active CD and UC (7). In addition, high levels of IFN-γ mRNA were found in freshly isolated lamina propria mononuclear cells (LPMN) from patients with active CD (1,8,9). IFN-γ mRNA concentrations also were increased in mucosal biopsies from active CD (1). Several studies have reported unchanged or even decreased production of IL-4 in UC (10-13). Interestingly, when lamina propria CD4+ cells were isolated from UC biopsies and stimulated in vitro, IL-5 production was increased (4). IL-5 is probably produced by highly differentiated Th2 cells no longer capable of produce IL-4, which may explain why we could detect very little IL-4 expression in UC specimens.

Our results are in accordance with findings in Th1-biased models of IBD that are characterized by increased production of IFN-γ (14-19). Moreover, neutralization of IFN-γ abrogated the established colitis in severe combined immunodeficient mice transferred with the CD45RB<sup>hi</sup> T-lymphocytes (20,21), whereas in the

**TABLE 1. Clinical findings of patient and controls**

<table>
<thead>
<tr>
<th>Disease</th>
<th>Controls</th>
<th>Ulcerative colitis</th>
<th>Crohn's disease</th>
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<tbody>
<tr>
<td>Age (mean)</td>
<td>32 (17-54)</td>
<td>32.8 (17-54)</td>
<td>54.7 (14-79)</td>
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<tr>
<td>Sex ratio</td>
<td>m:6</td>
<td>4:6</td>
<td>3:5</td>
</tr>
<tr>
<td>Disease history</td>
<td>6.2 (1-19)</td>
<td>5.4 (0.25-15)</td>
<td>4</td>
</tr>
<tr>
<td>Localization</td>
<td>ileum</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>colon</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>both</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Steroids</td>
<td>7</td>
<td>7</td>
<td>8</td>
</tr>
<tr>
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<td>8</td>
</tr>
<tr>
<td></td>
<td>mild</td>
<td>5</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>active</td>
<td>3</td>
<td>8</td>
</tr>
<tr>
<td>Surgery</td>
<td>10</td>
<td>8</td>
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</table>


FIG. 1. IFN-γ+ cells in the lamina propria of Crohn’s disease (CD) specimens. **Top left:** a diffuse infiltration of cells with macrophage and lymphocyte morphology (magnification x250). **Lower left:** a higher power view of lamina propria IL-4+ cells of the same CD specimens (x500). IFN-γ+ cells in lamina propria and submucosal layers in a UC specimen. **Top right:** The architecture of the mucosa is completely altered and infiltration of inflammatory cells is confined to the muscularis mucosae (x250). **Lower right:** infiltration of cells with basophil and lymphocyte morphology (x250) confined to the lamina propria compartment.

TABLE 2. Microscopic score of T cell infiltration

<table>
<thead>
<tr>
<th></th>
<th>Epithelium</th>
<th>Lamina Propria</th>
<th>Submucosa</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>mean</td>
<td>median</td>
<td>mean</td>
</tr>
<tr>
<td><strong>Anti-CD3</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>control</td>
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<td>0.5</td>
<td>2</td>
</tr>
<tr>
<td>CD</td>
<td>0.875</td>
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<td>2.7</td>
</tr>
<tr>
<td>UC</td>
<td>0.05</td>
<td>0.5</td>
<td>2.7</td>
</tr>
<tr>
<td><strong>Anti-CD8</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>control</td>
<td>1.3750</td>
<td>1</td>
<td>1.375</td>
</tr>
<tr>
<td>CD</td>
<td>1.125</td>
<td>1</td>
<td>1.4</td>
</tr>
<tr>
<td>UC</td>
<td>0.876</td>
<td>1</td>
<td>1.5</td>
</tr>
</tbody>
</table>

p < 0.001

SEM: standard error of mean
TNBS-induced colitis, anti-IL-12 decreased IFN-γ production and abrogated established disease (22).

Indeed, T-cell modulation and inhibition of Th1 responses have become a primary therapeutic goal of CD. Depletion of CD4+ T cells with an anti-CD4 monoclonal antibody (cMT-412) in 12 steroid refractory CD patients caused a dose-dependent reduction of circulating CD4+, reduced the CDAI (Crohn’s disease activity index) (23), and reversed the abnormal expression of major histocompatibility complex class II DR on the intestinal epithelium (24). Because human leucocyte-associated-class II expression by gut epithelial cells is up-regulated by IFN-γ, this finding suggests that anti-CD4 antibody treatment down-regulates T-lymphocyte activation.

Treatment of steroid-refractory Crohn’s patients with a single dose of anti-TNF-α antibody has resulted in
important therapeutic effects (25–27). These findings suggest that targeting cytokines that are involved in Th1 responses may be beneficial in CD. In particular, because of its proximal effects on Th1/Th2 differentiation, IL-12 is a promising candidate for the treatment of active CD and for the maintenance of the anti-TNF-α-induced remission. In summary, our findings support a predominant Th1 response in CD but not in UC.

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REFERENCES