Resistance of Crohn's Disease T Cells to Multiple Apoptotic Signals Is Associated with a Bcl-2/Bax Mucosal Imbalance

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Crohn’s disease (CD) is a condition characterized by excessive numbers of activated T cells in the mucosa. We investigated whether a defect in apoptosis could prolong T cell survival and contribute to their accumulation in the mucosa. Apoptotic, Bcl-2+/Bax− cells in tissue sections were detected by the TUNEL method and immunohistochemistry. T cell apoptosis was induced by IL-2 deprivation, Fas Ag ligation, and exposure to TNF-α and nitric oxide. TUNEL+ leukocytes were few in control, CD, and ulcerative colitis (UC) mucosa, with occasional CD68+ and myeloperoxidase+, but no CD45RO+, apoptotic cells. Compared with control and UC, CD T cells grew remarkably more in response to IL-2 and were significantly more resistant to IL-2 deprivation-induced apoptosis. CD T cells were also more resistant to Fas- and nitric oxide-mediated apoptosis, whereas TNF-α failed to induce cell death in all groups. Compared with control, CD mucosa contained similar numbers of Bcl-2+, but fewer Bax+, cells, while UC mucosa contained fewer Bcl-2+, but more Bax+, cells. Hence, the Bcl-2/Bax ratio was significantly higher in CD and lower in UC. These results indicate that CD may represent a disorder where the rate of T cell proliferation exceeds that of cell death. Insufficient T cell apoptosis may interfere with clonal deletion and maintenance of tolerance, and result in inappropriate T cell accumulation contributing to chronic inflammation.

**Materials and Methods**

**Study population**

Fifty-seven control patients (36 males and 21 females, ranging from 7 to 88 yr of age; 46 with colonic cancer, five with diverticular disease, four with familial polyposis, one with rectal hemangioma, and one with Hirschsprung’s disease), 41 CD patients (19 males and 22 females, ranging from 17–72 yr of age), and 47 UC patients (24 males and 23 females, ranging from 8 to 66 yr of age) were studied. Among IBD patients, 36 were receiving corticosteroids, 37 corticosteroids and aminosalycilates, seven were receiving only aminosalycilates, and 8 were receiving no specific treatment. All diagnoses were confirmed by clinical, radiological, endoscopic, and histological criteria.

**Evaluation of apoptosis**

Apoptotic cells were detected by in situ DNA end labeling (TUNEL method) (21) using the ApopTag Plus kit (Oncor, Gaithersburg, MD). Briefly, fixed tissue sections (0.5 × 2–3 cm) from surgically resected control and inflamed colons were deparaffinized by heating for 10 min at 70°C and rehydrated by subsequent exposures to xylene; 100, 95, and 70% ethanol; and PBS. Endogenous peroxidase was inactivated with 100% methanol containing 0.3% hydrogen peroxide for 5 min at room temperature. Sections were then treated with 20 μg/ml proteinase K (Sigma, St. Louis, MO) for 15 min at 37°C, immersed in equilibration buffer, and incubated with TdT, an enzyme that catalyzes the multiple 3′-OH ends generated by DNA fragmentation, and digoxigenin-labeled nucleotides in a humidified atmosphere at 37°C for 60 min. The reaction was terminated with stop/wash buffer, sections were incubated with anti-digoxigenin peroxidase for 30 min at room temperature, and stained with 3,3′-diaminobenzidine tetrahydrochloride (DAB). Apoptotic cells were counted in five random high power fields and expressed as the number of brown-stained nuclei or apoptotic bodies per 1000 methyl green-counterstained cells. Negative controls included sections treated with the TdT buffer solution omitting the TdT enzymes.

To identify the phenotype of the apoptotic cells a double-staining technique was employed in parallel tissue sections to allow for a direct comparison of the TUNEL method (purple color) with immunohistochemistry (brown color) for markers of lymphocytes, macrophages, and neutrophils. After the endogenous peroxidase inactivation step, sections were reacted with the mAb UCHL-1 (anti-CD45RO), PG-M1 (anti-CD68), or the polyclonal anti-myeloperoxidase Ab MPO-7 (all from Dakopatts, Glostrup, Denmark) for 12 h at 4°C and immunostained with DAB for 3–6 min at room temperature. The procedure continued with treatment by proteinase K and exposure to TdT, and thereafter cells were covered with anti-digoxigenin-alkaline phosphatase for 30 min at room temperature and stained with 5-bromo-4-chloro-3-indol phosphate/nitroblue tetrazolium chloride.

Apoptotic death of cell suspensions was monitored using a dye mix containing 100 μg/ml of acridine orange and 100 μg/ml of ethidium bromide (both from Sigma Chemical Co.) in PBS (22). The degree of apoptosis was quantified by an apoptotic index, calculated as the percentage of apoptotic cells divided by the number of total cells. In each culture at least 200 cells were counted by two observers blind to the cell source. Interobserver variation averaged 1%.

Apoptosis was also assessed by DNA fragmentation determined by agarose gel electrophoresis. DNA was prepared from T cells as described with some modification (23). In brief, T cells were washed in PBS; pelleted in Eppendorf tubes; resuspended in 500 μl of lysing buffer consisting of 10 mM EDTA, and 1× SSC, containing sodium lauryl sarcosinate and proteinase K (both from Sigma); and incubated at 50°C for 2 h. The samples were precipitated with ethanol, mixed with dye, and loaded into wells of a 0.5% agarose gel containing 5 μg/ml of ethidium bromide in electrophoresis buffer.

**Identification of Bcl-2- and Bax-expressing cells**

Immunohistochemical staining for Bcl-2 and Bax was performed with an Ag retrieval system as previously reported (24). The tissues derived from normal uninfamed colonic mucosa and IBD patients were fixed and processed respectively in brief. dewaxed tissue sections (0.5 × 2–3 cm) from resected inflamed colons were treated with 0.3% hydrogen peroxide in absolute methanol to inhibit peroxidase activity and then heated in a microwave in 10 mM sodium citrate buffer (pH 6.0). After blocking background staining with normal goat serum, sections were incubated overnight with a monoclonal anti-Bcl-2 Ab (1/100 dilution; Dakopatts) or polyclonal anti-Bax Ab (1/500 dilution; Pharmingen, San Diego, CA) at 4°C. Color was developed using a streptavidin-biotin detection kit (Dakopatts) with diaminobenzidine as the chromogen, and sections were counterstained with methyl green or hematoxylin. Cells were counted as described for the TUNEL method. As a negative control, the primary Ab was omitted in each sample.

**Establishment of T cell lines**

Three endoscopic biopsies were obtained from the mucosa of randomly selected patients using the same size biopsy forceps. All biopsies from CD and UC patients were obtained from inflamed mucosa. Biopsies were rinsed and incubated in HBBS with 2.5% penicillin-streptomycin-fungizone mixture for 4–5 h at room temperature to prevent bacterial contamination. Following a previously described protocol (25), each biopsy was cut into four equal pieces, yielding 12 fragments. Each fragment was put into a 24-CA well containing 1 ml of RPMI 1640, 20% fetal bovine serum (BioWhittaker, Walkersville, MD), penicillin-streptomycin-fungizone-gentamicin mixture, and 20 U/ml of IL-2 (Chiron, Emeryville, CA). Cultures were fed twice weekly by removing 50% of the volume and replacing it with fresh medium containing 40 U/ml of IL-2. Cell growth was invariably observed by the end of the first week of culture. At 2 wk, residual tissue fragments were removed from each culture, and all cells were pooled, counted using the trypan blue dye exclusion test, adjusted at 106 cells/ml, and transferred to a 12-well plate. Cells were fed with fresh medium and IL-2, and growth was followed at weekly intervals. At the end of each week, cell concentration was adjusted at 106 cells/ml.

**Flow cytometric analysis**

The cell surface markers of mucosal T cells were determined by flow cytometry using a Becton Dickinson FACS (Mountain View, CA). The following murine mAbs were used either as fluorescein or PE conjugates: anti-CD3 (T cells), CD4 (Th cells), CD8 (cytotoxic T cells), CD14 (monocytes), CD19 (B cells), CD25 (IL-2R α-chain), CD45RO (leukocytes), CD28 (T cells), and CD80 (B7-1), CRC β, TCR γδ (all from Becton Dickinson Immunocytometry Systems, San Jose, CA), and CD95 (Apo-1/Fas Ag, UB-2, Kamiya Biomedical, Tukwila, WA). The background level of immunofluorescence was determined by incubating cells with fluorescein or PE-conjugated mouse Ig.

**Induction and modulation of apoptosis**

Initial attempts to measure apoptosis of freshly isolated cells showed that reliable evaluation was impossible due to the presence of residual dead cells and less than optimal cell purity. Therefore, experiments were conducted using mucosal T cell lines derived from mucosal biopsies as detailed above, which were kept in the exponential growth phase by feeding them with IL-2 twice a week. After 2 wk at the time of IL-2 supply, cells were instead washed twice to remove any residual IL-2 and then incubated in medium alone for up to 48 h. Under such IL-2 deprivation conditions, T cell apoptosis is readily induced (26). In some cultures, IL-10 (R & D Systems, Minneapolis, MN; 10 ng/ml) or CTLA-4Ig fusion protein (10 μg/ml) was added to modulate apoptosis. In addition to IL-2 deprivation, apoptosis was induced by anti-Fas Ab, TNF-α, and NO. For Fas-mediated apoptosis, T cells (1 × 106 cells in 1 ml of a 48-well plate) and plate and incubated with anti-human Fas Ab (CH-11, Kamiya Biomedical) at 100 μg/ml at 37°C. After 24 h, apoptosis was evaluated by the ethidium bromide/acidine orange-staining method described above. For TNF-α- and NO-induced apoptosis, cells (1 × 106 cell/100 μl) were plated in wells of a 96-well plate plate and incubated with increasing concentrations of TNF-α (R & D Systems) or the NO donor S-nitrosoglutathione (GSNO; Sigma) (27) at 37°C. After 24 h, viability was evaluated with the MTS assay (28) by pulsing the cells with 20 μl of a mixture of MTS (Promega, Madison, WI) with phenazine methosulfate (Sigma) for the last 4 h of incubation and assessing cell change at 490 nm. Cell viability was calculated according to a standard curve and exhibited an excellent correlation with that of the ethidium bromide/acidine orange method. Glutathione and glutathione (both from Sigma) were used as NO donor controls for GSNO.

**RT-PCR**

The presence of B7-1-specific message was determined by PCR of cDNA generated from RT of cellular RNA fractions. PCR reactions (50 μl final volume) were incubated in Taq polymerase buffer (Stratagene, Menasha, WI), containing 20 mM of each dNTP, 100 pmol of the indicated primers, template (cDNA synthesized from 100 ng RNA), and 5 U of Taq polymerase (Stratagene). Reactions were incubated on a thermocycler (Stratagene) for 25 cycles consisting of 30 s at 94°C, 1 min at 55°C, and 1 min at 70°C. The forward and reverse primers for B7-1 DNA amplification were CTGAACTGACCCATTACGTC and CTGCGGAGACTGGTATA.
CAG, respectively defining a 480-bp DNA fragment (29). PCR products were run on 1% agarose gels, transferred to nylon membranes, and probed with a 32P-labeled human B7-1 cDNA probe (provided by Dr. P. S. Linsley, Bristol-Meyers Squibb, Seattle, WA).

Analysis of data

For the patterns of mucosal T cell growth, the primary outcome variable was the rate of change in cell count from 2 to 3 wk ((wk 3 − wk 2)/1) (25). The Wilcoxon rank sum test or signed rank test for paired data were used to test the differences in slopes for two groups, and the Kruskal-Wallis test was used for multiple groups. Results of repeated measures ANOVA using the actual cell counts for each week were consistent with the nonparametric tests, recognizing potential violations of the assumptions of normality and equal variance. For the time course of the number of viable or apoptotic cells (percent viability and apoptotic index, respectively) in the cell cultures the repeated measures ANOVA was used with SPSS for Windows 6.1 (SPSS, Chicago, IL). This type of analysis directly compares the variation of the chosen parameters (percent viability and apoptotic index) over the entire length of time of each experiment. To assess the rate of cell death in the suspensions, viable cells in individual cultures were enumerated over the time course, and the observations from each culture were fitted the equation $N(t) = N_0 \exp(-\delta t)$, where $N_0$ is the number of cells suspended at the start of the experiment, and $\delta$ is the death rate in h$^{-1}$ (30). For evaluation of nonparametric data, the Mann-Whitney rank-sum test was used. For all other statistical analysis, the paired $t$ and Wilcoxon’s signed-rank tests, for parametric and nonparametric data, were used, respectively. All results were expressed as the mean ± SEM. Statistical significance was inferred when $p < 0.05$.

Results

Frequency and phenotype of apoptotic cells in the mucosa

Apoptotic cells were infrequent in the lamina propria of control, CD, and UC tissue sections (four colonic specimens each). TUNEL$^+$ cells represented 1.32 ± 0.81% in control, 0.37 ± 0.24% in CD, and 4.55 ± 1.3% in UC of the total number of leukocytes. The number of apoptotic cells in CD mucosa was significantly ($p < 0.02$) smaller than that in UC mucosa, with no other significant differences observed. When double staining was conducted to identify the phenotype of apoptotic cells, they were all either CD68$^+$ or MPO$^+$ (Fig. 1, A and B). TUNEL$^+$/CD68$^+$ and TUNEL$^+$/MPO$^+$ cells were more frequent in UC sections. No apoptotic cells were identified in the CD45RO$^+$ population of control, CD, or UC sections (Fig. 1C).

Patterns of mucosal T cell growth

To investigate whether intestinal T cells from control, CD, and UC mucosa had an intrinsically different capacity for growth and expansion, mucosal biopsies from randomly selected subjects were cultured with IL-2. Under identical culture conditions, striking differences were noted among control, CD, and UC mucosa in regard to both cell number and growth kinetics. Compared with control, biopsies from CD mucosa generated more T cells whose growth peaked at 3 wk, whereas T cells from UC biopsies grew less and peaked at 2 wk (Fig. 2). The number of T cells from UC was significantly smaller than that from control and CD cells at 2, 3, and 4 wk. Because actively inflamed IBD mucosa contains more T cells than normal mucosa, we investigated whether the observed differences depended on the original number of cells in the biopsies or the adjustment of cell concentration at 10^6/ml at 2 wk and thereafter. To do so, the slope of each growth curve (between 2–3 wk) was analyzed using the rank-sum method. This revealed that the slopes of normal, CD, and UC T cells were significantly different ($p < 0.001$), indicating that the growth of T cells in each group varied independently of the number of T cells originally present in the cultures and the cell concentration adjustment performed each week. In addition, when the slopes of the growth curves were compared using repeated measures analysis, the overall responses of normal, CD, and UC T cells to IL-2 were still significantly different ($p < 0.001$). Drug therapy (corticosteroids or aminosalicylates) had no influence on cell growth in IBD cultures or cell surface marker expression (see below).

Phenotype of mucosal T cell lines

The marked differences detected in regard to the growth kinetics of control, CD and UC T cell cultures could be due to the differential expansion of various subpopulations of mononuclear cells in response to IL-2. To investigate this possibility, we characterized the phenotype of T cells after 2 wk in culture using a large panel of surface Ags (Table I). The phenotype of the T cells was remarkably similar regardless of diagnosis. All cultures were essentially...
FIGURE 2. Distinct growth rates of mucosal T cells in response to IL-2. T cell lines were established from mucosal biopsy fragments cultured in the presence of human rIL-2 (20 U/ml). Number of experiments: 36 control, 26 CD, and 26 UC. Lines between 0 and 2 wk are broken to indicate that no measurements were performed during this time interval. *, p < 0.03 for CD compared with UC at 2 wk and for UC compared with control at 3 and 4 wk, respectively; **, p < 0.02 for CD compared with UC at 4 wk and for UC compared with control at 2 wk; ****, p < 0.005 for CD compared with UC at 3 wk.

pure T cells (CD3+) composed predominantly by memory (CD45RO+) and helper (CD3+ CD4+ CD8-) rather than cytotoxic (CD3+ CD4- CD8+) cells and were virtually free of monocytes (CD14+) and B cells (CD19+). The number of cells expressing activation markers (CD25 and HLA-DR) was also comparable in all cultures. T cells expressed almost exclusively the TCRab.

Finally, all cultures contained comparably high levels of the Apo1/Fas Ag at similar levels of expression.

IL-2 deprivation-induced apoptosis of mucosal T cells

Because the differences observed in the growth of control, CD, and UC T cells could not be explained by phenotypically diverse cell populations, we studied their survival in culture by assessing viability and the rate of apoptosis. After IL-2 was removed from the cultures, CD T cells displayed no loss in viability for a full 24-h period, after which a decline started (Fig. 3A). In contrast, control and UC T cells began dying as early as 3 h and continued to do so steadily for the remainder of the cultures. The rate of cell death in the CD cultures (δ = 0.0020 ± 0.0004 h⁻¹) was significantly (p < 0.01) slower than that in the control cultures (δ = 0.0034 ± 0.0003 h⁻¹), and also slower (p < 0.05) than that in the UC cultures (δ = 0.0034 ± 0.0005 h⁻¹). The time course of percent viability in CD cultures was significantly different from that in control (p < 0.001) and UC cultures (p < 0.001), but the time courses of percent viability in the control and UC cultures were not different (p = 0.89). In the same cultures the increase in the rate of apoptotic index of CD T cells was clearly slower compared with that in control and UC cultures (Fig. 3B). This was confirmed by the apoptotic index of CD cells, which was significantly smaller than that of control (p < 0.04) and UC (p < 0.006) cells, whereas the apoptotic indexes of control and UC cells were not different (p = 0.735). These findings were confirmed by DNA gel analysis. When tested after 12 h of IL-2 deprivation, DNA from control and UC cells showed a ladder-like pattern characteristic of apoptosis, which was not observed in the CD cultures. The viability of CD cells is significantly different from that of control (p < 0.001) and UC (p < 0.001) cells, but the viability of UC is not different (p = 0.89) from that of control cells. The time course of the apoptotic index of CD cells is significantly different from that of control (p < 0.04) and UC (p < 0.006) cells, but the time course of the apoptotic index of UC is not different (p = 0.735) from that of control cells.

Table 1. Phenotype of intestinal mucosal T cell linesa

<table>
<thead>
<tr>
<th>Marker</th>
<th>Control</th>
<th>CD</th>
<th>UC</th>
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<tbody>
<tr>
<td>CD45RO+</td>
<td>97.1 ± 0.9</td>
<td>98.1 ± 1.6</td>
<td>96.0 ± 1.7</td>
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<td>CD3+</td>
<td>94.7 ± 1.6</td>
<td>97.7 ± 2.5</td>
<td>93.7 ± 2.6</td>
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<tr>
<td>CD3+ CD4+ CD8-</td>
<td>77.6 ± 4.6</td>
<td>81.5 ± 6.0</td>
<td>80.0 ± 6.1</td>
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<tr>
<td>CD3+ CD4+ CD8+</td>
<td>9.0 ± 4.1</td>
<td>8.6 ± 4.2</td>
<td>12.5 ± 5.5</td>
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<tr>
<td>CD3+ CD25+</td>
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<td>31.9 ± 2.7</td>
<td>34.3 ± 7.6</td>
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<td>CD3+ HLA-DR+</td>
<td>73.2 ± 1.8</td>
<td>68.9 ± 3.3</td>
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<tr>
<td>TCRαβ+</td>
<td>91.4 ± 2.2</td>
<td>95.2 ± 3.6</td>
<td>92.7 ± 8.9</td>
</tr>
<tr>
<td>TCRγδ+</td>
<td>1.1 ± 0.6</td>
<td>1.4 ± 0.5</td>
<td>2.4 ± 1.5</td>
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<td>CD14+</td>
<td>0.2 ± 0.1</td>
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<td>0.2 ± 0.1</td>
</tr>
<tr>
<td>CD19+</td>
<td>0.4 ± 0.1</td>
<td>0.9 ± 0.3</td>
<td>0.9 ± 0.4</td>
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<tr>
<td>CD95+</td>
<td>99.5 ± 0.3</td>
<td>98.5 ± 0.6</td>
<td>98.2 ± 0.8</td>
</tr>
<tr>
<td>CD95 MFIb</td>
<td>112.5 ± 4.8</td>
<td>96.9 ± 8.7</td>
<td>94.8 ± 8.0</td>
</tr>
</tbody>
</table>

Values indicate % positive cells as assessed by flow cytometric analysis. Number of experiments: 22 control, 18 CD, and 18 UC.

a Mean fluorescence intensity.
whereas DNA of CD T cells revealed no fragmentation (Fig. 4). At the end of the cultures (48 h) the degree of DNA fragmentation was comparable in all cultures. These results were independent of the drugs used by the patients.

**IL-2 production by mucosal T cells and effect of IL-10 on apoptosis**

Next, we explored the possibility that the observed differences in apoptotic rate were due to production of autocrine growth factors, such as IL-2. When exogenous IL-2 was removed, no endogenous IL-2 was detected by a sensitive bioassay in any of the cultures (31). We also investigated whether control, CD, and UC T cells differed in their ability to respond to cytokines that protect from apoptosis, such as IL-10 (32). The addition of IL-10 had a protective effect in all cultures, but this varied among control, CD, and particularly UC cells (Fig. 5). When cell decay rates were calculated in the absence and the presence of IL-10, there was a significant ($p < 0.005$) effect of diagnosis; the decay rate of CD cultures ($\delta_1 = 0.0021 \pm 0.0006$, $\delta_2 = 0.0011 \pm 0.0005 \, h^{-1}$, respectively) was smaller than that of either control ($\delta_1 = 0.0035 \pm 0.0006$ and $0.0025 \pm 0.0007 \, h^{-1}$) or UC ($\delta_1 = 0.0046 \pm 0.0001$ and $0.0045 \pm 0.0012 \, h^{-1}$) cultures. These results are consistent with the findings above (Fig. 3A). The effect of IL-10 in slowing the rate of apoptosis over time was significant ($p < 0.001$) in each diagnostic group. Thus, the decreased apoptosis observed in CD cultures was independent of the effect of IL-10.

**Fas-, TNF-α-, and NO-induced apoptosis of mucosal T cells**

To learn whether the decreased apoptosis of CD mucosal T cells was a phenomenon only observed when essential growth factors were withdrawn or reflected a global, trigger-independent resistance to apoptosis, alternate mechanisms of cell death were explored. When apoptosis was induced by ligation of the Fas Ag with the CH-11 Ab, the apoptotic index of CD, but not UC, mucosal T cells was significantly lower ($p < 0.006$) than that of control cells (Fig. 6). In contrast, ligation of the Fas-related TNF receptor with TNF-α failed to induce death of mucosal T cells in any group regardless of the dose used (Fig. 7A). Finally, exposure to GSNO-derived NO induced a dose-dependent loss of viability of all mucosal T cells, but loss was significantly reduced in CD compared with control T cells ($p < 0.02$), while the decrease in UC T cell viability essentially overlapped with that in control cells (Fig. 7B). The GSNO-related, non-NO donors glutathione and oxidized glutathione compounds caused no cell death when used at the same concentrations of GSNO (data not shown).

**CD28/CTLA-4:B7 costimulatory pathway and effect of CTLA-4Ig**

Because the mucosal T cell cultures were free of accessory cells and endogenous IL-2, other growth stimuli could only derive from

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**FIGURE 4.** DNA gel electrophoresis of IL-2-deprived mucosal T cell lines. Fragmentation was assessed after 12 h of IL-2 deprivation using the experimental conditions described in Fig. 3. One microgram of DNA was subjected to electrophoresis on a 1.5% agarose gel and stained with ethidium bromide. From left to right, laddering of DNA fragments of 200-bp multiples characteristic of apoptosis is noted in the first (C, control) and third (UC), but not in the second (CD) lane. The fourth represents the m.w. (MW) ladder. This figure is representative of four different experiments.

**FIGURE 5.** Inhibitory effect of IL-10 on T cell line apoptosis under conditions of IL-2 deprivation. Apoptosis was assessed using the experimental conditions described in Fig. 3. After washing, each cell line was split in two and cultured in the absence (−) and presence (+) of human rIL-10 (10 ng/ml). Number of experiments: A, six control; B, six CD; C, six UC. In all three panels the effect of IL-10 is significant ($p < 0.001$) for each diagnostic group.

**FIGURE 6.** Fas-mediated apoptosis of mucosal T cells. T cell lines were exposed to an optimal concentration of the anti-Fas Ab CH-11 for 24 h. after which the apoptotic index was calculated using the acridine orange/ethidium bromide dye mix staining method. Number of experiments: 11 control, 15 CD, and 11 UC. *, $p < 0.006$ for CD compared with control.
the T cells themselves. One possibility is signaling through the costimulatory CD28/CTLA-4-B7 pathway, which is known to be expressed on activated T cells (33). After 2 wk in culture, control and CD T cells expressed similar proportions of CD8$^+$ and B7-1$^+$ cells, as shown by flow cytometric analysis (Table II). In contrast, both markers were expressed by a significantly smaller number of UC than control or CD cells. The presence of B7-1 on T cells was corroborated by the detection of specific transcripts in RNA extracts by RT-PCR (data not shown). To investigate whether the CD28/B7-1 costimulatory pathway was involved in modulation of mucosal T cell apoptosis, the CTLa-4Ig fusion protein, which inhibits the interaction of CD28 and CTLA-4 with their ligands, was added to the cultures at the beginning of IL-2 deprivation. This resulted in no change in the apoptotic index of control, CD, or UC cultures.

Expression of anti- and pro-apoptotic proteins in the intestinal mucosa

Finally, we investigated whether the resistance of CD mucosal T cells to apoptosis could be related to some abnormality in the Bcl-2 family of proteins, whose balance is essential to cell death and survival (35). Mucosal sections were immunohistochemically stained for the anti-apoptotic Bcl-2 and the pro-apoptotic Bax proteins, the number of positive cells was counted, and the ratio of Bcl-2$^+$/Bax$^+$ cells was calculated. Lamina propria mononuclear cells expressed Bcl-2, while Bax was expressed by mononuclear and epithelial cells (Fig. 8, A–C). Both the number and the relative proportion of Bcl-2$^+$ and Bax$^+$ cells were markedly different in control, CD, and UC mucosa (Fig. 9, A–C). The number of Bcl-2$^+$ cells was similar in control and CD mucosa, but UC mucosa contained significantly fewer Bax$^+$ cells compared with control (p < 0.01) and CD (p < 0.001) mucosa (Fig. 9A). Opposite results were observed with regard to Bax. Compared with the control, the number of Bax$^+$ cells was significantly lower in CD (p < 0.002) and greater in UC (p < 0.03) mucosa; the difference between CD and UC was statistically significant (p < 0.001; Fig. 9B). Most importantly, the Bcl-2/Bax ratio was dramatically and significantly higher in CD compared with both control (p < 0.002) and UC (p < 0.001) mucosa (Fig. 9C). In contrast, this ratio was significantly lower in UC compared with control mucosa (p < 0.01).

**Table II. Expression of B7-1 and CD28 by intestinal mucosal T cell lines**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>CD</th>
<th>UC</th>
</tr>
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<tbody>
<tr>
<td>B7-1</td>
<td>63.4 ± 2.1</td>
<td>67.0 ± 3.4</td>
<td>49.3 ± 4.6*</td>
</tr>
<tr>
<td>CD-28</td>
<td>83.7 ± 2.0</td>
<td>81.9 ± 3.1</td>
<td>73.8 ± 4.2**</td>
</tr>
</tbody>
</table>

* Values indicate % positive cells as assessed by flow cytometric analysis. Number of experiments: 10 control, 10 CD, and 8 UC.

* p < 0.05; ** p < 0.02 compared with both control and CD cells.

**Discussion**

Immune homeostasis and self tolerance are complex events that depend on signals from the Ag receptor, costimulatory molecules, accessory cells, and growth factors (36). These events are dynamically integrated with apoptosis, resulting in a balance that limits T cell responses (12). Because there is uncontrolled T cell activation in IBD (3, 4, 8), abnormalities of homeostatic mechanisms of apoptosis might be expected in CD or UC.

The detection of apoptotic cells in the gastrointestinal tract has been investigated using various techniques, varying from morphological criteria (37), labeling of fragmented nuclear DNA (21), to staining of proteins involved in the regulation of cell death (38). In contrast to these studies, which were directed at the rapidly dividing population of epithelial cells, our study was specifically designed to assess apoptosis in mucosal immune cells. Apoptotic leukocytes were rare in the lamina propria and were exclusively CD68$^+$ and MPO$^+$, indicating a selectivity in the type of cells locally undergoing cell death. The increased number of TUNEL$^+$ cells in UC was largely due to dying neutrophils, a short-lived cell that is abundant in this condition. Somewhat surprising was the absence of apoptotic CD45RO$^+$ memory T cells, even in areas actively involved by IBD. However, the same finding is noted in other tissues involved in chronic inflammation, such as the synovium in rheumatoid arthritis and muscle fibers in inflammatory myopathies (39, 40). Even in T cell-mediated animal models of IBD, such as the trinitrobenzenesulfonic acid-induced colitis in mice, apoptotic lymphocytes are virtually undetectable in actively inflamed mucosa (I. Fuss, unpublished observations). Taken together, these observations suggest that the paucity of apoptotic T cells is a characteristic of immune-mediated chronic inflammatory conditions. The paucity of apoptotic cells in vivo, however, may be deceptive, because detection of apoptotic bodies is a fleeting phenomenon and even a minute number of them indicates a substantial rate of cell turnover (41). This may explain apparent discrepancies between in vivo and in vitro results and underscores the importance of studies that bring out functional differences between normal and diseased T cells and analytical methods measuring cell death over time (30).

The composition of lymphoid cells in normal and IBD mucosa is well known. It does not consist of grossly distinct lymphocyte populations, but, rather, of equivalent proportions of T cells, T cell subsets, B cells, and macrophages (9, 42) with an increased expression of activation markers (3, 4, 8) and a predominant memory phenotype (43, 44). Thus, at the beginning of the cultures all mucosal biopsies contained similar populations of T cells and accessory cells. Furthermore, mucosal T cells were TUNEL$^+$, as demonstrated by the in situ TdT labeling. Phenotypic analysis showed that expansion in IL-2 resulted in pure T cells, devoid of monocytes and B cells in all groups. Differences might have been detected if additional markers were studied. However, we aimed our analysis at demonstrating that possible differences in apoptosis among control, CD, and UC mucosal T cells were not due to contamination by other cell types. This homogeneity eliminates the
influence of lymphocyte subsets and costimulation between different cell types that may mask the intrinsic susceptibility of mucosal T cells to apoptosis (45). Mature T cells are susceptible to activation-driven cell death (46), and growth factors such as IL-2 modulate their apoptosis (23, 26). Thus, the greater growth of CD, compared with control and UC, T cells in response to IL-2 does not simply reflect the presence but also the type of inflammation (CD vs UC), confirming our previous findings with mucosal T cells.

FIGURE 8. Immunohistochemical staining identifying cells expressing the anti-apoptotic Bcl-2 and the pro-apoptotic Bax proteins in colonic intestinal lamina propria. A, Control; B, CD; C, UC. Micrographs were taken from sequential tissue sections of representative tissue specimens (original magnification, ×200).
from a pediatric population of IBD patients (25). Moreover, the resistance of CD T cells to apoptosis contrasted with the equal susceptibility of control and UC cells and cannot be explained by differences in IL-2 utilization, since control and IBD lymphocytes consume comparable amounts of this cytokine (31). Resistance to IL-2 deprivation-induced apoptosis is also exhibited by rheumatoid arthritis T cells (47), leading to speculate that this defect may be a characteristic of chronic diseases in which T cells play a major pathogenic role. There is emerging evidence that removal of reactive T cells after Ag engagement is mainly due to passive death caused by growth factor deprivation (36). Loss of this feedback mechanism could lead to T cell overexpansion, particularly in the Ag-rich environment of the gut, a possibility supported by our results.

Because CD mucosal T cells displayed a defective apoptosis upon IL-2 deprivation, other modulators of programmed cell death might also induce a distinct response in this condition. To investigate this possibility, control, CD, and UC T cells were cultured with IL-10, a cytokine that promotes survival of IL-2-dependent T cells (54). We have recently confirmed the existence of this imbalance in CD (61). These observations are consistent with similar findings in rheumatoid arthritis T cells (17, 45) and help strengthen the emerging concept that defective T cell apoptosis is a central event in immune-mediated inflammatory diseases.

In addition to cytokines, several other factors determine T cell survival or death. Among them, the Fas (APO-1/CD95) and Fas ligand system is particularly relevant to regulation of mucosal immunity because continuous Ag stimulation renders T cells susceptible to Fas-mediated apoptosis and induces persistent Fas ligand expression (36, 48). Continuous Ag exposure is the norm for mucosal T cells, and probably explains why they routinely express both Fas and Fas ligand and display a greater degree of Fas-mediated apoptosis than blood T cells (49, 50). Thus, the decrease in Fas-mediated apoptosis of T cells in CD becomes especially important to pathogenesis, because failure of this death pathway can impair both autocrine and paracrine mechanisms for elimination of activated T cells (51, 52). Because Fas is member of the TNF receptor family (53), differences in TNF-α-mediated apoptosis could be expected among control, CD, and UC mucosal T cells, but even at very high concentrations TNF-α failed to induced apoptosis. This is probably due to the fact that the cells we tested were CD4⁺ T cells, and TNF-α only induces death of CD8⁺ T cells (54).

In addition to death mediated by specific receptors, mucosal T cells can also die through nonspecific mechanisms, such as oxidative stress. This form of apoptosis is mediated by either reactive oxygen metabolites or NO (20, 55), whose levels are greatly increased in actively inflamed IBD mucosa (56, 57). Our results showing that CD T cells are significantly resistant to NO-mediated apoptosis further extends the spectrum of death-inducing agents to which CD T cells are less susceptible. This is important because it expands understanding of mechanisms of inflammation in IBD and helps differentiate CD from UC. The decreased susceptibility to apoptosis regardless of the type of death signals implies that T cells may be intrinsically resistant to cell death in CD, a phenomenon apparently not involved in UC pathogenesis.

To investigate other mechanisms contributing to the enhanced proliferation or longer survival of CD T cells, we analyzed the costimulatory CD28/CTLA-4:B7 system. CD28 is constitutively expressed on T cells, while CTLA-4 is induced after activation, and they bind to their respective counter-receptors B7-1 (CD80) and B7-2 (CD86) (58). The high growth and low apoptosis of CD mucosal T cells could be due to an enhanced B7-1 expression, but this was not so based on the results of the phenotypic analysis. In contrast, a significantly smaller number of UC cells expressed CD28 and B7-1 than control and CD T cells. Therefore, while the greater growth and survival of CD mucosal T cells could be due to defective apoptosis, the smaller growth of UC mucosal T cells could be related to limited CD28-B7 costimulation. When the inhibitory protein CTLA-4Ig was used (59), it failed to affect the degree of apoptosis in all cultures. One explanation could be that circulating and mucosal T lymphocytes are differentially regulated at the level of the CD28/CTLA-4:B7 pathway, as CTLA-4Ig does not influence TCR-mediated apoptosis (60).

The results discussed above demonstrate that all pathways of mucosal T cell apoptosis we explored are impaired in CD. The multiplicity of this defect strongly suggests that fundamental regulatory mechanisms controlling T cell death and survival are abnormal in this type of IBD. The family of Bcl-2-related proteins constitutes the most relevant class of apoptosis regulatory gene products (35). More important than the level of expression of individual anti- or pro-apoptotic proteins is the relative proportion of death antagonists to agonists, and this ratio determines how a cell will respond to an apoptotic signal (35). Immunostaining of mononuclear cells for Bcl-2-related proteins showed multiple abnormalities of both Bcl-2 and Bax in CD and UC mucosa. However, the most striking observation was that the Bcl-2/Bax ratio was remarkably increased in CD, skewing the balance in favor of cell survival. We have recently confirmed the existence of this imbalance in CD by flow cytometric studies with purified lamina propria T cells (61). These observations are consistent with similar findings in rheumatoid arthritis T cells (17, 45) and help strengthen the emerging concept that defective T cell apoptosis is a central event in immune-mediated inflammatory diseases.

**FIGURE 9.** Quantitative analysis of mononuclear cells expressing the anti-apoptotic Bcl-2 and pro-apoptotic Bax proteins in colonic intestinal lamina propria. Positive cells were identified by immunohistochemical staining of seven control, eight CD, and 10 UC tissue specimens. A. Bcl-2: *, p < 0.01 for UC compared with control; **, p < 0.001 for UC compared with CD. B. Bax: *, p < 0.002 for CD compared with control; **, p < 0.001 for CD compared with UC; ***, p < 0.03 for UC compared with control. C. Bcl-2/Bax ratio: *, p < 0.002 for CD compared with control; **, p < 0.001 for CD compared with UC; ***, p < 0.01 for UC compared with control.
Suicidal cell death is a complex series of events regulated by the intrinsic propensity of individual cells, cellular environment, cell surface molecules, type and timing of activation, growth factors, and multiple oncogene products (62). Overall, this study suggests that CD represents a disorder of T cell accumulation, in which the rate of cell proliferation exceeds that of cell death (13). Cell death is the physiological response of T cells to high Ag concentrations, and it is aimed at eliminating reactive T cells and attenuating immune reactivity (63). Nowhere in the body is the Ag concentration higher than that in the gut, where a tight control of T cell apoptosis is expected. Mucosal T cells apparently escape this regulatory mechanism in CD, extending the life span of Ag-primed T cells and retaining activated cells otherwise destined to die. This would interfere with clonal deletion and the development of tolerance (64), resulting in an exceedingly strong or prolonged immune response predisposing to chronic mucosal inflammation.

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References