American Journal of Pathology, Vol. 152, No. 1, January 1998

Activated CD4$^+$ and CD8$^+$ Cytotoxic Cells Are Present in Increased Numbers in the Intestinal Mucosa from Patients with Active Inflammatory Bowel Disease

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The contribution of cell-mediated cytotoxicity to the pathogenesis of inflammatory bowel disease (IBD) is controversial, and results of *in vitro* assays vary according to experimental procedures. Therefore, we compared the frequency of cytotoxic effector cells *in situ*. On tissue sections of controls (n = 11), low frequencies of granzyme A and perforin mRNA-expressing cells are found in the lamina propria (1.77 ± 0.15% and 1.46 ± 0.12%, respectively) and in the epithelial cell layer (0.76 ± 0.12% and 0.66 ± 0.10%, respectively). In patients with IBD (n = 33), corresponding values were significantly ($P < 0.02$) higher, 6.1 ± 0.40% and 5.92 ± 0.57% for granzyme A and perforin expression in the lamina propria and 2.50 ± 0.19% and 2.59 ± 0.28%, respectively, in the epithelial compartment. Differences between ulcerative colitis and Crohn's disease are statistically not significant ($P > 0.33$). Activated cytotoxic cells are preferentially found at sites facing the intestinal lumen. Perforin mRNA-expressing cells are mainly CD8$^+$ T cells. CD4$^+$ T cells expressing perforin mRNA are mainly isolated from affected areas of patients with Crohn's disease. Immunostaining for perforin protein generally coincides with perforin mRNA *in situ*. These data demonstrate that cytotoxic cells are vigorously activated *in situ* in the intestinal mucosa of patients with active IBD. *(Am J Pathol 1998, 152:261–268)*

Recently, support has been provided for the notion of an aberrant regulation of the local immune response as a major factor in the pathogenesis of inflammatory bowel disease (IBD). The role of cell-mediated cytotoxicity in the pathogenesis of Crohn's disease and ulcerative colitis, however, is controversial. Although peripheral T cells from patients with IBD show elevated cell-mediated cytotoxic activity when assayed in a redirected lysis assay *in vitro*,[1] drastically reduced natural killer (NK) cell activity was found in the peripheral blood of patients with Crohn's disease[2] or in patients with either Crohn's disease or ulcerative colitis.[3] On the other hand, several of the recently characterized gene knockout mice that develop colitis display an increased cell-mediated cytotoxic activity of the intestinal lamina propria lymphocytes (LPLs) and intraepithelial lymphocytes (IELs).[4–9]

Our understanding of the molecular mechanisms involved in cell-mediated cytotoxicity has dramatically improved over the past few years. Experiments carried out with both naturally occurring and experimentally derived gene-deficient mice led to the elucidation of at least two major pathways of cell-mediated cytotoxicity. The first pathway requires the exocytosis of several proteins including the pore-forming protein perforin and one or several members of a serine protease family called granzymes. The genes coding for granzymes and perforin are generally expressed only after specific activation; hence the detection of cells containing transcripts of these genes is often used as a means to localize cytotoxic cells (CTLs, NK cells) activated *in situ*.[10] The second mechanism of cell-mediated cytotoxicity is operative even in the absence of calcium and can be attributed mainly, if not completely, to a Fas-mediated induction of apoptosis in target cells.[11] Fas-mediated cytolysis seems to be a common effector mechanism in Th1 CD4$^+$ T cells,[12] whereas in cytotoxic CD8$^+$, Th2 CD4$^+$, and NK cells exocytosis of perforin- and granzyme-containing cytoplasmic granules appears to account for most of the observed cytotoxic activity.[13]

The controversy on the contribution of cell-mediated cytotoxicity in patients with IBD[1,2,3,14] may at least par-

Supported by grant 31-43495.95 from the Swiss National Science Foundation (to C. Mueller).

Accepted for publication October 10, 1997.

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tially be due to 1) different procedures used to isolate cells from affected lesions, 2) variations in different *in vitro* assay systems used to measure cell-mediated cytotoxicity, 3) the processing of tissue samples, 4) therapeutic treatments of patients, and 5) analysis of peripheral blood cytotoxic cells *versus* LPLs or IELs. To circumvent some of the problems associated with an *in vitro* analysis of cell-mediated cytotoxicity, we decided to assess the contribution of activated cytotoxic cells to the tissue destruction in IBD by *in situ* hybridization for perforin and granzyme A mRNA.[10] Immunohistochemical staining for the detection of human perforin was performed in parallel to confirm the presence of perforin expression also on the protein level. To further obtain information on the cellular source of these perforin mRNA-expressing cells, isolated cells of the lamina propria and the epithelial cell layer from affected tissue specimens were sorted on a cytofluorometer according to their cell surface phenotypes for subsequent *in situ* hybridization.

Materials and Methods

Tissue Specimens for In Situ *Hybridization on Frozen Section*

Twenty tissue specimens from seven patients with Crohn's disease (CD; three men and four women; mean age, 36 years) and thirteen specimens from eight patients with ulcerative colitis (UC; five men and three women; mean age, 48 years) were obtained after surgical resection of the bowel. In the group with CD, the sites of disease were ileal (n = 2), ileocolonic (n = 4), and colonic (n = 1). Indication for bowel resection was the presence of strictures or fistulae. In the group with UC, all patients showed signs of a severe pancolitis, refractory to medical management. Oral corticosteroids had been administered to all patients with the exception of one with CD and one with UC. Control tissues (five specimens from the small bowel and six from the colon) were obtained from patients with colon cancer (n = 4), stomach cancer (n = 1), pancreatitis (n = 1), and diverticular disease of the colon sigmoideum (n = 1). In patients with cancer, macroscopically normal small intestinal and colonic samples were chosen in areas remote from neoplastic lesions. Blood supply was interrupted approximately 30 to 45 minutes before the specimens were sampled. Tissue specimens were snap frozen immediately after removal and stored at −70°C, and adjacent tissue specimens were processed for conventional diagnostic histopathological assessment.

Tissue Specimens for IEL and LPL Isolation

Native tissue specimens of at least 10 cm^2 were collected from macroscopically affected areas as described above from four patients with CD (all samples from ileum; three women and one man; mean age, 49 years) and three patients with UC (three women; mean age, 30 years). Small bowel control tissue specimens were obtained from the terminal ileum of two patients who underwent right hemicolectomy for colonic carcinoma and one multi-organ donor. Large bowel tissue specimens were obtained from two patients with colon carcinoma after hemicolectomy and one multi-organ donor.

Preparation of Cryostat Sections

Five-micron cryostat sections of the intestinal tissue samples were placed on poly-L-lysine-coated glass slides (Menzel Gläser, Braunschweig, Germany) and fixed in phosphate-buffered saline (PBS)-buffered 4% paraformaldehyde for 20 minutes. After dehydration through graded ethanol, the slides were stored at 4°C for several weeks until they were used for *in situ* hybridization.

Preparation of ^{35}S-Labeled RNA Probes

An 800-bp fragment of the human granzyme A cDNA (HF gene)[15] (generously provided by I. L. Weissman, Stanford) and a 1953-bp cDNA fragment of the human perforin gene[16] (kindly provided by J. Tschopp, Lausanne), cloned into the expression vectors pGEM-1 and pBluescript, respectively, were used to prepare ^{35}S-labeled RNA probes using T7 or SP6 RNA polymerase reactions, as previously described.[17]

In Situ *Hybridization*

In situ hybridizations of cryostat sections and single-cell suspensions were done as previously described.[17] After hybridization and washing off nonhybridized probe, slides were dipped into NTB-2 emulsion (Eastman-Kodak, New Haven, CT) diluted 1:2 in 800 mmol/L ammonium acetate. Slides were exposed in the dark at 4°C for 28 days. Slides were developed with Kodak developer PL-12 for 5 minutes and fixed with Kodak fixer for 10 minutes at room temperature. Counterstaining was done either with nuclear fast red (0.05% in 5% aluminum sulfate) or with hematoxylin. From each tissue, sections were hybridized with the ^{35}S-labeled sense probe of either the granzyme A gene or the perforin gene (2×10^5 cpm/μl hybridization solution) as a negative control.

Evaluation of Tissue Sections and Cell Preparations

Cells were considered positive for expression of the granzyme A or perforin gene when they had at least twice as many silver grains as the cells with the highest background on control slides that were hybridized with a labeled sense probe sequence identical to the cellular target mRNA. No evidence for a nonspecific binding of the radiolabeled RNA probes was found.

To determine the frequency of perforin- and granzyme-A-expressing cells on a tissue section, 10 fields (0.11 mm^2 each) were randomly selected and the frequency of cells expressing the perforin (or the granzyme A) gene was determined in the epithelial cell layer and in the lamina propria. To determine whether or not activated

cytotoxic cells are equally distributed within the intestinal crypts, 10 crypts were randomly chosen and divided into three zones of equal length with zone C being at the base of the crypts and zone A representing the top third of the crypts facing the gut lumen. Again, frequencies of perforin or granzyme A mRNA-containing cells in the epithelial cell layer and the lamina propria were determined separately.

Immunohistochemistry

Perforin was detected immunohistochemically on cryostat sections using biotinylated mouse anti-human perforin monoclonal antibody (MAb) clone 2d4.[18] Tissue sections were fixed in acetone and rehydrated in PBS containing 10% horse serum for 10 minutes. Primary antibody was diluted in PBS containing 1% bovine serum albumin at a working concentration of 5 μg/ml. After incubation at room temperature for 45 minutes, slides were washed three times in PBS for 5 minutes for subsequent incubation with avidin-FITC conjugate (Southern Biotechnology, St. Louis, MO) for 30 minutes at room temperature. Slides were embedded for immunofluorescence microscopy in 33% Ficoll 400 (w/v) in 50 mmol/L Tris containing 1 mg/ml *p*-phenylenediamine. A biotinylated mouse MAb (14.4.4) specific for mouse I-A was used as an isotype-matched negative control.

Isolation of IELs and LPLs

For the isolation of IELs and LPLs, the intestinal mucosa was first dissociated mechanically from the other layers of the intestinal wall and teased into small pieces in cold Hanks' balanced salt solution (HBSS) containing 2% horse serum. The epithelium was separated from the lamina propria by an incubation with 2 mmol/L dithiothreitol containing 0.1 mmol/L EDTA in HBSS plus 2% horse serum at 37°C for 30 minutes. Cells were subsequently passed first through a 70-μm and then a 40-μm cell strainer (Becton Dickinson, San Jose, CA) and centrifuged for subsequent purification on a Percoll gradient (Pharmacia Biotech, Uppsala, Sweden). Remaining tissue was treated three times with 5 mmol/L EDTA in HBSS plus 5% horse serum for 15 minutes at 37°C with intermittent vortexing. Tissues were subsequently washed in HBSS plus 5% horse serum and HBSS plus 5% horse serum containing Ca^{2+}/Mg^{2+}, followed by an incubation with DNAse I (Boehringer Mannheim, Mannheim, Germany)/collagenase type IV (Sigma Chemical Co., St. Louis, MO) for 25 minutes at 37°C on a magnetic stirrer. This incubation step was repeated twice, and supernatants with isolated lamina propria cells were pooled, passed through a 70- and 40-μm cell strainer, and centrifuged for subsequent purification on a Percoll gradient. To this end, pellets (lamina propria cells or cells of the intestinal epithelium) were each resuspended in 7 ml of 38% Percoll and underlaid with 3 ml of 73% Percoll. The 73%/38% Percoll gradient was overlaid with 3 ml of 19% Percoll and centrifuged at 900 × *g* for 15 minutes at room temperature. Cells in the lower interphase were collected, washed, and resuspended for staining.

In Situ *Hybridization of Isolated $CD4^+$ and $CD8^+$ Cell Subsets*

Isolated IELs and LPLs were stained for 15 minutes on ice with directly conjugated antibodies against CD4 and CD8α (PharMingen, San Diego, CA). After completion of the staining procedure, cells were fixed in freshly prepared 4% buffered paraformaldehyde for 10 minutes and resuspended in PBS. A FACSVantage (Becton Dickinson) was used for the separation of $CD4^+$ and $CD8^+$ cell subsets after gating on the lymphoid cell population in the forward and side light scatter of the isolated IEL or LPL population. Cells were directly sorted onto poly-L-lysine-coated microscope slides (Menzel Gläser), controlled by Pascal-based Lysys II software (Becton Dickinson). Phenotypic analysis was performed on a FACScan using Macintosh-based CellQuest software (Becton Dickinson). *In situ* hybridization of sorted cells was performed as described above.

Statistics

For statistical analysis, the unpaired Student's *t*-test was used; *P* values of <0.05 were considered statistically significant.

Results

Activated Cytotoxic Cells in the Intestinal Epithelial Cell Layer and the Lamina Propria

In situ hybridization of control intestinal tissues reveals a frequency of less than 1% for cells expressing the granzyme A and perforin gene in the epithelial cell layer. Control tissue samples from the large bowel show slightly higher frequencies of perforin and granzyme A mRNA-expressing cells than specimens from the small bowel

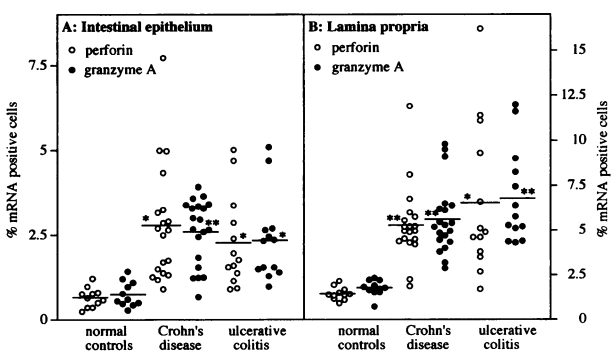

Figure 1. Scattergram showing frequencies of cells with detectable mRNA of the genes coding for perforin (○) and granzyme A (●) in the epithelial cell layer (**A**) and the lamina propria (**B**) of tissue samples from IBD and controls. Differences between samples from patients with CD and UC are statistically not significant ($P > 0.33$). Differences between CD or UC and normal controls are statistically significant for perforin and granzyme A expression in intestinal epithelium and lamina propria, (—, mean; $^*P < 0.02$; $^{**}P < 0.01$).

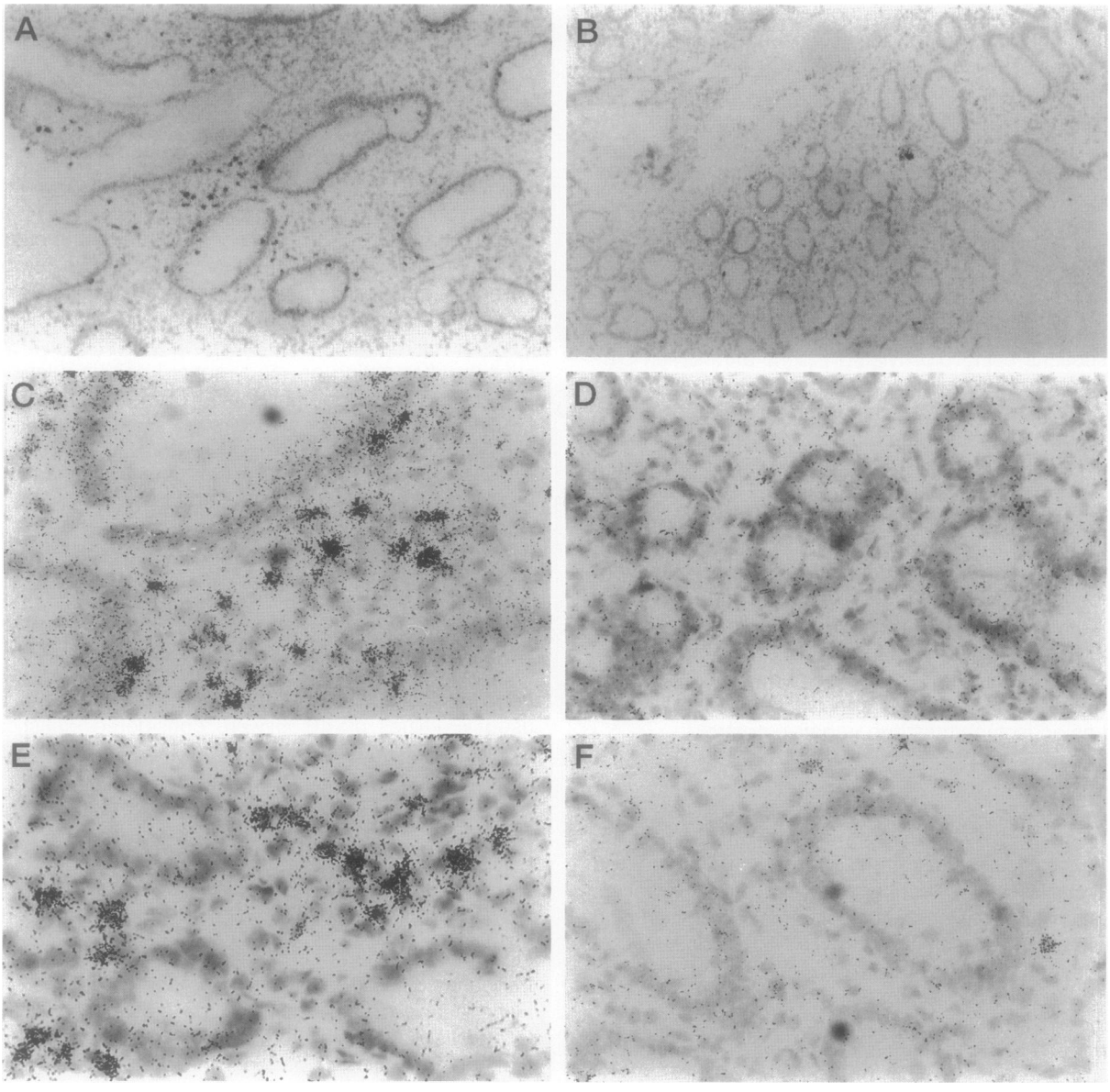

Figure 2. *In situ* hybridization of cryostat sections from colon of a patient with ulcerative colitis (**A, C,** and **E**) and from a normal colon (**B, D,** and **F**) using ^{35}S-labeled antisense mRNA probes of the granzyme A (**A** to **D**), and the perforin gene (**E** and **F**). Magnification, ×75 (**A** and **B**) and ×300 (**C** to **F**).

(0.90 ± 0.17% and 0.58 ± 0.15%, respectively, for granzyme A mRNA expression and 0.83 ± 0.11% and 0.44 ± 0.10%, respectively, for perforin mRNA expression). Compared with the frequencies observed in the control group, values obtained from resected intestinal specimens from patients with active IBD show a significant, approximately threefold increase in the proportion of cells expressing the granzyme A ($P < 0.01$ for both CD and UC) or the perforin gene ($P < 0.02$ for both CD and UC) in the epithelial cell layer (Figure 1A).

For the lamina propria, *in situ* hybridization with gene probes of the granzyme A and perforin mRNA essentially reveals a similar pattern as for the epithelial cell layer. In the lamina propria of patients with IBD, cells expressing granzyme A or perforin mRNA are more than threefold increased compared with the intestinal lamina propria in the control group. These differences are statistically significant, both for the frequency of granzyme A mRNA-expressing cells ($P < 0.01$ for both CD and UC) and perforin mRNA-expressing cells ($P < 0.01$ for both CD and UC). Almost identical values are obtained for the number of cells expressing granzyme A or perforin (Figure 1B). Representative results of *in situ* hybridizations for the detection of perforin and granzyme A mRNA-expressing cells in tissue sections from patients with active IBD and from control patients are shown in Figure 2. Frequencies of cells expressing either perforin or granzyme A mRNA in both the epithelial layer and the lamina propria do not differ significantly ($P > 0.33$) on tissue sections from patients with CD compared with patients with UC.

Immunohistochemical detection of perforin-containing cells in the lamina propria and the epithelial cell layer revealed essentially an identical distribution pattern as found for mRNA-containing cells (Figure 3). Perforin-pro-

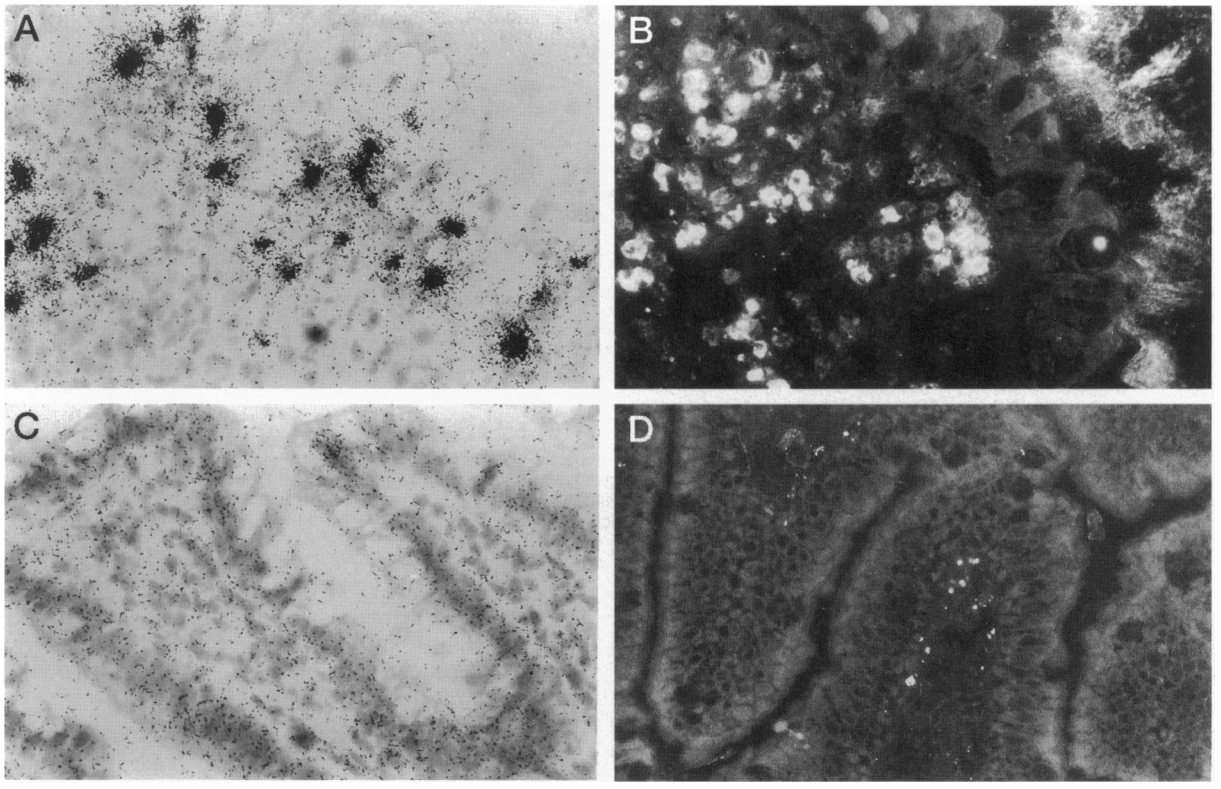

Figure 3. Comparative analysis of perforin mRNA (**A** and **C**) and perforin protein (**B** and **D**) expression, detected by *in situ* hybridization, using ^{35}S-labeled perforin antisense mRNA, and immunofluorescence staining, using FITC-conjugated anti-human perforin MAb, on sections from the terminal ileum of a patient with CD (**A** and **B**) and from a control patient (**C** and **D**). Magnification, ×300.

tein-containing cytotoxic cells are preferentially found at sites where the epithelial cell layer shows histopathological signs of deterioration (data not shown).

Activated Cytotoxic Cells Are Preferentially Located in the Upper Parts of the Crypts Facing the Intestinal Lumen in Active IBD

To further study the distribution pattern of perforin and granzyme A gene expression in IBD, 10 crypts on each section were divided into three zones of equal length as described in Materials and Methods, and the frequency of perforin and granzyme A gene-expressing cells was re-evaluated for each zone. The results of this re-evaluation are depicted in Figure 4 for granzyme-A-expressing cells. For perforin, an almost identical distribution pattern is seen as for granzyme-A-expressing cells within intestinal crypts, both in the epithelial cell layer and the lamina propria, *ie*, decreasing frequencies of perforin- and granzyme-A-expressing cells from the top toward the base of the crypts. In the outermost zone A, perforin- and granzyme-A-expressing cells are twice as frequent as in the middle zone B. In the zone closest to the base (zone C), perforin- and granzyme-A-expressing cells are again only half as frequent as in the middle zone B.

Perforin-Expressing Cells Are Preferentially Found in CD8$^+$ and CD4$^+$ Cell Subsets

To better characterize the T cell compartment in the intestinal mucosa of the tissue specimens used to isolate IELs and LPLs, the CD4:CD8 ratio was determined first. In patients with UC, the ratio of CD4$^+$ to CD8$^+$ IELs is generally increased compared with normal large bowel (mean, 1.86:1 *versus* 0.31:1, respectively). However, due to large variations in the frequencies of CD4$^+$ and CD8$^+$

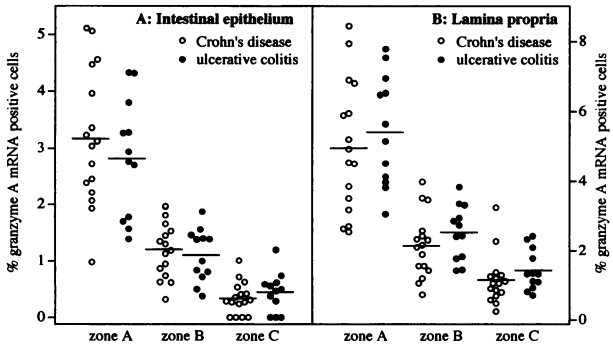

Figure 4. Scattergram showing frequencies of granzyme A mRNA-positive cells in the epithelial cell layer (**A**) and the lamina propria (**B**) of different zones of the crypts in tissue specimens from patients with CD (○) and UC (●); zone A, outermost zone facing the gut lumen; zone B, middle zone; zone C, basal zone of the crypt (—, mean).

Table 1. Frequencies of $CD4^+$ and $CD8^+$ T Cells Isolated from the IEL and LPL Compartments of Patients with Active IBD and Controls

	CD4	CD8	CD4:CD8
A: IEL compartment			
Controls			
Small bowel (n = 3)	31.9 ± 1.5	53.7 ± 8.3	0.62 ± 0.10
Large bowel (n = 3)	18.2 ± 4.4	61.7 ± 5.2	0.31 ± 0.10
IBD			
Crohn's disease (n = 4)	34.2 ± 2.6	55.1 ± 8.9	0.72 ± 0.18
Ulcerative colitis (n = 3)	51.9 ± 5.1*	37.5 ± 10.4	1.86 ± 0.87
B: LPL compartment			
Control			
Small bowel (n = 3)	53.2 ± 7.7	24.0 ± 5.6	2.34 ± 0.29
Large bowel (n = 3)	64.7 ± 4.3	26.0 ± 3.0	2.52 ± 0.15
IBD			
Crohn's disease (n = 4)	62.1 ± 3.6	38.3 ± 5.7	1.74 ± 0.28
Ulcerative colitis (n = 3)	62.8 ± 1.9	26.5 ± 3.9	2.48 ± 0.37

Results are presented as the relative frequencies of $CD4^+$ and $CD8^+$ T cells in the IEL and the LPL compartment in percent of total isolated $CD45^+$ cells (mean ± SE); n = number of individuals analyzed. For statistical analysis, data obtained from patients with ileal CD and UC were compared with data obtained from tissue specimens of normal small and large bowel respectively.
*$P < 0.01$.

IELs in the group of patients with UC, this difference is statistically not significant ($P = 0.17$). With the exception of the phenotypic composition of IELs from patients with UC in the present study, no obvious differences in the proportions of $CD4^+$ and $CD8^+$ cells of the intestinal mucosa are observed between patients with UC or CD and normal large or small bowel, respectively (Table 1). Generally, larger numbers of lymphocytes were isolated from tissue specimens of patients with active IBD, compared with normal controls, particularly from the lamina propria (data not shown).

The results of the *in situ* hybridization with radiolabeled RNA antisense probe of the perforin gene of isolated $CD4^+$ and $CD8^+$ T cells, respectively, are shown in Figure 5. In the $CD8^+$ cell subsets, less than 10% (8.1 ± 0.9%) and in the $CD4^+$ cell subset, less than 2.5% (1.9 ± 0.2%) of the cells express perforin mRNA in IELs or LPLs of the small and large bowel in control patients. In patients with CD, frequencies of $CD4^+$ and $CD8^+$ cells expressing perforin mRNA are significantly ($P < 0.05$) increased compared with the values obtained with normal controls in both the IEL and the LPL compartment. Higher frequencies of perforin mRNA-expressing IELs and LPLs are also found in patients with UC compared with normal controls, although, with the exception of $CD8^+$ IELs ($P < 0.05$), these increases are not statistically significant ($P > 0.1$). The highest frequencies of cells containing perforin mRNA at detectable levels is found in the $CD8^+$ cell subset of the lamina propria from patients with CD (36.1 ± 7.7%). Most remarkably, in the $CD4^+$ subset of the lamina propria, which represents the major lymphoid cell population in this compartment of patients with CD, the fraction of perforin mRNA-expressing cells is increased fivefold (10.9 ± 2.9%) compared with controls (1.9 ± 0.2%). In the epithelial cell layer of patients with CD, frequencies of perforin-expressing $CD8^+$ and $CD4^+$ T cells (24.3 ± 4.5% and 7.3 ± 1.2%, respectively) are slightly below the values obtained with lamina propria T cells. In patients with UC, the frequencies of perforin mRNA-expressing cells in the $CD8^+$ and $CD4^+$ IEL subsets (18.2 ± 2.3% and 5.5 ± 2.6%, respectively) and LPL (17.5 ± 3.5%, and 4.4 ± 2.3%, respectively) are generally lower than those observed in the corresponding cell populations from patients with CD.

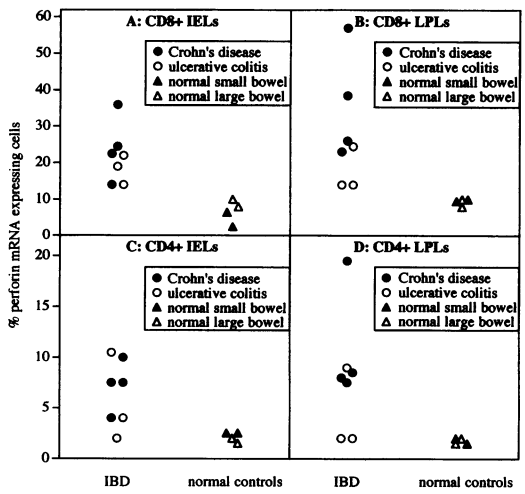

Figure 5. Frequencies (in percent) of perforin-expressing cells in $CD8^+$ (**A** and **B**) and $CD4^+$ (**C** and **D**) IELs (**A** and **C**) and LPLs (**B** and **D**) from control patients (△ and ▲) and from patients with active IBD (○ and ●). Mean values and standard errors are indicated in the main text. Differences are statistically significant ($P < 0.05$) between diseased tissue and corresponding normal controls for all cell subsets isolated from patients with CD and for $CD8^+$ IELs from patients with UC.

Discussion

Reports on the cell-mediated cytotoxic activity of T cells and NK cells in patients with IBD and normal controls are inconclusive so far. Evidence for an increased, unaltered, or even decreased cell-mediated cytotoxic activity (assessed in *in vitro* assays) of T cells or NK cells isolated form patients with IBD compared with cells from normal controls has been provided.[1–3,14] In this study we compare the extent of activation of potentially cytotoxic T cells

directly in intestinal lesions of patients with IBD with normal control tissues through the detection of transcripts of the perforin and the granzyme A gene by *in situ* hybridization.

Taking this approach, we demonstrate an important activation of cytotoxic effector cells in the intestinal mucosa of patients with IBD. The frequency of activated, perforin- and granzyme-A-expressing cells is equally increased in tissue specimens from patients with active ulcerative colitis and Crohn's disease. The frequency of perforin or granzyme A mRNA-expressing cells is increased severalfold in the intestinal mucosa of patients with IBD compared with controls; activated cytotoxic cells, however, are not evenly distributed in the affected mucosa. This points toward the importance of analyzing resection specimens rather than biopsy specimens of the intestinal mucosa. The study of the latter, in fact, might even lead to slightly different results due to a sampling error of the tissue. In the lamina propria and the epithelial cell layer, the relative increase in the frequency of perforin and/or granzyme A mRNA-containing cells is comparable. However, the difference in the cellular composition of the two compartments does not allow for a direct comparison of these results. Considering the increased cellularity in the lamina propria in tissues from patients with IBD, the observed differences in the frequencies of activated cytotoxic cells between controls and patients with IBD become even more relevant. Activated cytotoxic cells are preferentially localized in the outermost part of crypts both in patients with IBD (Figure 4) and in controls (data not shown). Although our results do not allow for a conclusive explanation of this finding, it appears likely that elevated access to antigens from the gut lumen and/or enhanced antigen-presenting capacities of epithelial cells at these locations may lead to the observed distribution of activated cytotoxic cells within the intestinal mucosa. In addition to their cytolytic properties, activated cytotoxic cells may further affect the pathogenesis of IBD through the secretion of cytokines, in particular of interferon-γ which may further favor the generation of a cell-mediated immune response. The cytotoxic effects of activated cytotoxic cells on epithelial cells as target cells may turn on a vicious circle: epithelial cells are reported to produce various cytokines with pro-inflammatory or chemotactic properties such as interleukin-8 or epithelial neutrophil-activating peptide 78 (ENA-78).[19–23] Thus, destruction of these epithelial cells may lead to the release of large amounts of stored cytokines, which in turn leads to a perpetuation of the inflammatory reaction. Circumstantial evidence for the storage of chemokines by epithelial cells has been recently found for the C-X-C chemokine ENA-78 that is detected by immunohistochemistry in a large fraction of intestinal epithelial cells in the absence of detectable amounts of specific mRNA.[24] Tissue samples from individual patients with IBD revealed a considerable variation in the frequencies of activated cytotoxic cells in the intestinal mucosa. These variations, however, cannot be solely attributed to differences in the therapeutic treatments of the patients with IBD; in fact, among the 14 patients with IBD used for the initial analysis of perforin and granzyme A gene expression on tissue sections, 2 patients (1 case with Crohn's disease and 1 case with ulcerative colitis) did not receive specific medication. Furthermore, the tissue samples analyzed from these two patients did not differ significantly in the frequency of activated, granzyme A or perforin mRNA-expressing LPLs or IELs compared with the patients with IBD that were under treatment at the time of surgery (data not shown).

The conflicting results in the literature on the relative contribution of different T cell subsets to cell-mediated cytotoxic activity in patients with IBD and in mouse models of IBD prompted us to assess the frequency of perforin and/or granzyme A mRNA-containing cells in the $CD4^+$ and the $CD8^+$ cell subset. Due to the different fixation requirements for *in situ* hybridization and immunostaining, we stained isolated cells from the lamina propria and the epithelial layer for subsequent cell sorting on a FACSVantage. Sorted cells were used for *in situ* hybridization. The results provide clear evidence that both $CD4^+$ and $CD8^+$ T cells are activated in the intestinal mucosa of patients with IBD to express the perforin gene at detectable levels. The observed high frequency of perforin mRNA-containing cells in $CD8^+$ cells raises the question about the nature of the antigen(s) involved in the activation of this T cell subset. It will be of particular interest to determine whether the large fraction of $CD8^+$ T cells activated *in situ* to express the granzyme A and perforin genes is the result of an oligoclonal expansion of antigen-reactive T cells *in situ* or whether a dysregulated immune response may lead to the activation of bystander cells irrespective of their antigen specificity. The fact that only a minor although, compared with controls, significantly increased subpopulation of $CD4^+$ T cells express the perforin gene does not imply that this subset is of minor importance in the cell-mediated cytotoxicity. $CD4^+$ T cells, primarily those of the functional Th1 phenotype, may also use a granzyme-A- and perforin-independent way of killing, *ie*, through Fas-Fas ligand (FasL) interactions. Although the relevance of the FasL-Fas pathway in the cytolysis mediated by $CD4^+$ T cells cultured short term *in vitro* could not be further substantiated in a recent report,[25] the relatively slow kinetics of target cell lysis mediated by $CD4^+$ T cells isolated from the lesions of interleukin-2 null mice with active disease[8] have been regarded as indirect evidence for a possible involvement of a perforin-independent pathway. On the other hand, Simpson et al[26] could demonstrate the same kinetics of colitis induction in tgε26 mice[9] after adoptive transfer of bone marrow derived from FasL defective *gld/gld* mice instead of normal bone marrow, further questioning the involvement of Fas-mediated cytotoxicity at least in a mouse model of colitis.

With regard to the developmental origin and the possible antigen specificity and major histocompatibility complex restriction of $CD4^+$ and particularly of $CD8^+$ cells expressing perforin mRNA, it will be of interest to further define their surface phenotype. The distinction between CD8$\alpha\beta$ and CD8$\alpha\alpha$ T cells might allow an assessment of the contribution of intrathymically derived and putatively extrathymically derived T cells. The latter subset might have undergone incomplete negative se-

lection as suggested by studies in T-cell-receptor transgenic mice and hence may include a considerable portion of potentially autoreactive T cells.

In conclusion, the increased frequencies of granzyme-A- and perforin-expressing cells in the intestinal mucosa of patients with active IBD indicate that, at least in these forms of IBD, effector cells of cell-mediated cytotoxicity are strongly activated. The co-localization of activated cytotoxic cells and preserved epithelial cells may indicate the involvement of cytotoxic cells in the destruction of epithelial cells. Our findings together with the recently reported observation of an increased interleukin-12 expression in the gut of patients with Crohn's disease[27,28] clearly demonstrates an important role of a Th1 shift and cell-mediated cytotoxicity in the pathogenesis of IBD. Local activation of CTLs may lead to the release of pro-inflammatory cytokines and chemokines from necrotic epithelial cells and thus further amplify the inflammatory reaction. Our results strongly indicate that cell-mediated cytotoxicity contributes to the exacerbation and perpetuation of active IBD.

Acknowledgments

We thank M. Jungo and I. Vogt for expert technical assistance, Dr. R. E. Hunger and Dr. J. A. Laissue for helpful discussion, and Dr. S. Birrer and Dr. H. E. Wagner for generous supply of tissue samples.

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