Defective Expression of gp180, a Novel CD8 Ligand on Intestinal Epithelial Cells, in Inflammatory Bowel Disease

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Abstract

Previous studies support a role for intestinal epithelial cells (IEC) as antigen-presenting cells in mucosal immune responses. T cells activated by IEC are CD8+, suppressor in function, and dependent upon CD8-associated p56lck activation. A 180-kD glycoprotein (gp180) recognized by mAbs B9 and L12 has been identified and shown to be important in CD8+ T cell activation by IEC. Since IEC derived from patients with inflammatory bowel disease (IBD) are incapable of activating CD8+ T cells, we asked whether this correlated with gp180 expression. While frozen sections of normal bowel revealed bright gp180 staining on all IEC, both inflamed and uninflamed ulcerative colitis (UC) specimens showed patchy staining. In Crohn's disease (CD), staining was faint to absent. Flow cytometry confirmed immunohistochemical data. The staining patterns correlated with the ability of IEC to activate CD8-associated p56lck. Normal IEC induced phosphorylation of p56lck in CD8α but not CD4+ transfectants. In contrast, both UC and CD IEC activated CD4 and, to a much lesser extent, CD8-associated p56lck. Thus, gp180 expression by IBD IEC appears to be altered, and correlates with a functional alteration of lck activation. This defect may reflect a more proximal event in the pathogenesis of IBD. (*J. Clin. Invest.* 1997. 100:2062–2071.) Key words: epithelium • CD8+ T cells • Crohn's disease • ulcerative colitis • glycoprotein

Introduction

The inflammatory bowel diseases (IBDs),[1] ulcerative colitis (UC) and Crohn's disease (CD), are characterized by chronic active inflammation in an organ where controlled inflammation is the norm. Work from several laboratories has supported the concept that this active inflammatory process is the result of a dysregulated immune response. More specifically, there is evidence of constitutively activated CD4+ T cells within the lamina propria that can leak into the systemic circulation (1, 2). These activated CD4+ T cells secrete a panel of cytokines that may promote localized cell-mediated granulomatous inflammation (CD) (3–6) or a more diffuse antibody- and complement-mediated Arthus-type reaction (UC) (7, 8). While many pathogenetic mechanisms have been proposed to explain these observations, to date no consistent infectious, neurohormonal, or immunologic mechanism has been elucidated. Despite this, there have been tremendous advances in our knowledge of these diseases. There is a growing consensus that IBD reflects the combination of an altered immune response to one of multiple luminal antigens in a genetically susceptible host. The nature of the altered immune response, the aberrant activation of CD4+ T cells in the lamina propria, remains to be determined.

Our laboratory has long been interested in the role of intestinal epithelial cells (IEC) in regulating mucosal immune responses. In vitro studies suggest that normal epithelial cells can activate CD8+ suppressor T cells, which may be relevant to the controlled inflammation seen in the intestine in vivo (9, 10). We have identified previously a molecule expressed on normal IEC that appears to play a role in the activation of antigen nonspecific CD8+ suppressor T cells (11, 12). This molecule, recognized by two mAbs, B9 and L12, is a 180-kD glycoprotein (gp180) that mediates binding to CD8 and results in the activation of the src-like tyrosine kinase p56lck (13). Activation of this kinase is necessary but not sufficient for the proliferation of CD8+ T cells induced by normal epithelial cells.

Previous studies from this laboratory have shown that there is a defect in the activation of suppressor cells by IEC derived from patients with IBD (14). These results are seen regardless of the site from which the epithelial cells are derived. That is, epithelial cells from both actively inflamed and uninvolved areas of IBD display similar defects. This suggests that there may be an inherent defect in all IBD epithelium that results in the failure of suppressor cell generation. When IBD epithelial cells are called upon to present antigen, they may do so in an aberrant fashion, resulting in the activation of CD4+ T cells with consequent chronic active inflammation. Nonimmune physical and chemical barriers, such as tight junctions and intestinal mucus, may prevent chronic inflammation from being a constant in these patients.

In this study, we show that defects in gp180 expression are found in IEC from UC and CD, and correlate with functional differences in the activation of CD8+ suppressor T cells. We hypothesize that the alterations in gp180 expression may lead potentially to a loss in the capacity of IBD IEC to mediate suppressor cell activation, allowing for the inflammation characteristic of IBD to persist.

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1. *Abbreviations used in this paper:* CD, Crohn's disease; CDI, inflamed intestine from patients with CD; CDU, uninflamed intestine from patients with CD; DIV, diverticulitis; gp180, 180-kD glycoprotein; IBD, inflammatory bowel disease; IEC, intestinal epithelial cells; NL, normal tissue; SCID, severe combined immunodeficiency; UC, ulcerative colitis; UCI, inflamed intestine from patients with UC; UCU, uninflamed intestine from patients with UC.

Methods

Specimens. Surgical specimens from patients undergoing bowel resection for cancer (10 cm away from tumor), CD, UC, or other inflammatory diseases [diverticulitis (DIV), ischemic colitis] at the Mount Sinai Medical Center were used as a source of epithelial cells. For flow-cytometric studies, endoscopic biopsies from normal and IBD patients undergoing diagnostic or surveillance colonoscopy were also used as epithelial cell sources. Samples were obtained from 16 normal patients (histologically and grossly normal areas of intestinal specimens derived from patients having colonoscopy or bowel resection for cancer and non-IBD inflammatory conditions) and 48 IBD patients. The presence or absence of inflammation in tissues was determined grossly and confirmed histologically.

Records of all patients were reviewed, and their histories and medical regimens noted. None of the patients were on high-dose immunomodulators (6-mercaptopurine, azathioprine) at the time of surgery. Their treatment regimens included 5-aminosalicylic acid alone, steroids alone, 6-mercaptopurine and steroids, or no therapy at all.

mAbs. mAbs B9 and L12 (both IgG1) against human intestinal epithelial cell membrane antigens were generated in our laboratory as previously described (11). Both antibodies recognize different carbohydrate side chains on gp180. This molecule appears to be involved in the activation of CD8+ T cells by normal IEC (12). Positive and negative controls for IEC staining used mAbs W6/32 (American Type Culture Collection, Rockville, MD) and IgG1 antidinitrophenol (the kind gift of Dr. Jay Unkeless, Mount Sinai Medical Center, New York), respectively.

Immunohistochemical studies. Tissue sections were stained by a well-described method (15). Bowel specimens were placed in cold calcium and magnesium–free HBSS (CMF HBSS; GIBCO BRL, Gaithersburg, MD) at the time of surgery and transported immediately to the laboratory. They were then oriented and snap-frozen in Tissue Tek (Miles Scientific Div., Miles Laboratories, Inc., Naperville, IL) and either stored at $-70°C$ or processed immediately for thin sectioning. Serial 4-μm-thick frozen sections were prepared and stored at $-70°C$ for staining. Indirect staining with B9 and L12 mAbs was performed using an avidin-biotin-peroxidase technique (ABC-Vectastain Kit; Vector Laboratories, Inc., Burlingame, CA), with Gill's hematoxylin (Fisher Scientific Co., Springfield, NJ) as a counterstain. All stained sections were reviewed blindly and independently by at least two investigators, including a trained pathologist. Discrepancies in interpretation were resolved by review of the slides by both investigators and at least one additional investigator. Criteria for positive staining were set before initiation of the studies and agreed to by all investigators. As a positive control, staining with the anti–class I mAb W6/32 was performed in each series of experiments. Each section was compared with a corresponding control consisting of an irrelevant isotype–matched antibody or no added antibody. No significant background staining was evident.

Isolation of epithelial cells. The procedure used to isolate epithelial cells has been described previously (16). Resected tissue specimens were rinsed extensively with PBS to remove loosely adherent material, and then rubbed gently with sterile gauze. The surface mucosa was stripped off from the underlying submucosa by careful dissection and minced into tiny pieces. These were then washed several times in RPMI 1640 containing 1% penicillin/streptomycin, 1% fungizone, 50 mg/ml gentamicin (all from GIBCO BRL) and 10 mM Hepes (Sigma Chemical Co., St. Louis, MO). The tissue was then treated with 1 mM DTT (Sigma Chemical Co.) in RPMI 1640 for 15 min at 22°C. After washing three times with RPMI 1640 containing antibiotics, the tissue pieces were incubated in RPMI 1640 containing 1 mg/ml dispase (Boehringer Mannheim Biochemicals, Indianapolis, IN) for 30 min at 37°C in an orbital shaker rotating at 150 rpm. During this treatment, epithelial cells and intraepithelial lymphocytes were released from the tissue. Cells that remained loosely adherent to the tissue were recovered using a rubber policeman. Dispase treatment was repeated once. The cell suspensions resulting from dispase treatments were washed twice, pelleted, and resuspended in a 15-ml

conical tube in 3 ml of 100% Percoll (Pharmacia Biotech, Piscataway, NJ) which had been adjusted to pH 7.4 with an osmolality of 290 mosM. 3-ml layers of 60, 40, and 30% Percoll (prepared by diluting 100% Percoll with RPMI 1640) were layered successively on top to establish a Percoll density gradient. The Percoll gradient was centrifuged at 1,500 rpm for 30 min at 4°C. Cells at the top 0/30% layer interface contained > 95% pure epithelial cells. Purity was confirmed by flow-cytometric analysis (PROFILE; Coulter Corp., Hialeah, FL) using anti-CD14, -CD3, -CD20 (Coulter Corp.), and L12 (anti–epithelial cell) mAbs. Contaminating cells were predominantly CD3+ T cells (2–4%). Cells were washed three times in RPMI, and viability was determined by trypan blue exclusion. Only cell preparations with a viability > 90% were used for staining and analysis in T cell–IEC cocultures. Preliminary studies using colonic epithelial cell lines documented that dispase treatment had no effect on the expression of gp180 as assessed by staining with mAbs B9 and L12.

Quantitation of gp180 in IEC. 10^5 freshly isolated IEC were washed three times with 1% BSA (Sigma Chemical Co.) in PBS, then incubated for 45 min with mAbs B9 or L12 previously conjugated to FITC (Sigma Chemical Co.) by a well-established method (17). Next, the cells were washed three times with 1% BSA-PBS and then analyzed by flow cytometry. Positive and negative controls for IEC staining used mAbs W6/32 and IgG1 antidinitrophenol, which had also been conjugated to FITC.

To further ensure the purity of our IEC preparation, two sets of analyses were performed. First, to eliminate possible lymphocyte contamination, IEC preparations were also incubated with KC56 (T-200)-RD1 (anti-CD45 mAb conjugated to phycoerythrin; Coulter Corp.). CD45+ cells were gated out during flow cytometry. In the second analysis, IEC preparations were permeabilized using Permea-Fix (Ortho Diagnostic Systems Inc., Raritan, NJ) and incubated with CAM 5.2 (anti–human cytokeratin mAb; Becton Dickinson, San Jose, CA) followed by goat anti–mouse Ig conjugated to R-phycoerythrin (Biosource International, Camarillo, CA). During flow cytometry, measurements of gp180 expression were determined only on cells positive for CAM 5.2. This second approach was more time-consuming than the first, but both approaches yielded similar data.

Preparation of IEC lysates. 10^6 freshly isolated epithelial cells were incubated in an Eppendorf tube on ice with cold lysis buffer (1% NP-40, 1 mM PMSF, 10 mM iodacetamide, and 1 mM Na$_3$VO$_4$ [all from Sigma Chemical Co.], in four parts PBS to one part distilled water) for 30 min, vortexing every 5 min. The lysate was centrifuged at 13,000 rpm for 10 min in a microfuge to remove cell debris.

Murine transfectants 3G4 and 3G8. The transfectants 3G4 and 3G8 are murine T cell hybridomas expressing human CD4 and CD8α antigens, respectively. They were kindly donated by Dr. S.J. Burakoff (Dana-Farber Cancer Institute, Boston, MA). Construction of these transfectants has been detailed previously. To produce the 3G4 cell line, the T cell hybridoma BY155.16 was transfected with the defective retrovirus MNST4 carrying full-length human CD4 cDNA, and murine transfectants expressing human CD4 antigen were selected by sorting (18). The 3G8 cell line was constructed in a similar manner, using the T cell hybridoma BY155.16 and the retroviral expression vector MNCT8 carrying full-length human CD8α cDNA (19).

Mixed T cell–IEC coculture. $0.5–1 \times 10^6$ 3G4 or 3G8 cells were incubated in an Eppendorf tube with equal numbers of normal or IBD IEC or antibodies cross-linking CD4 or CD8 in a 37°C water bath for varying time periods. At specific time points, the tube was transferred to ice, and 1 ml of ice-cold stop buffer (1 mM Na$_3$VO$_4$ in PBS) was added. The cells were then pelleted, and 100 ml of ice-cold lysis buffer (1 mM PMSF, 10 mM iodacetamide, and 1 mM Na$_3$VO$_4$, in four parts PBS to one part distilled water) was added for 30 min, vortexing every 5 min. Next, the lysates were centrifuged at 13,000 rpm for 1 min in a microfuge to remove cell debris.

Western blot with B9 or antiphosphotyrosine mAbs. IEC or mixed T cell–IEC lysates were resolved on 10% SDS-PAGE and transferred onto a nitrocellulose membrane at 15 V overnight in transfer buffer (20% methanol, 150 mM glycine, 25 mM Tris, pH 8.3). The transfer

sandwich was prewetted in transfer buffer and set up in the following order: (a) a porous polyethylene foam sheet; (b) one sheet of 3M filter paper; (c) the gel; (d) a nitrocellulose membrane sheet (0.2 μm) (Schleicher & Schuell, Inc., Keene, NH); (e) another sheet of 3M filter paper; and (f) another sheet of porous polyethylene foam. The transfer sandwich was inserted into a transfer electrophoresis unit (Hoefer Scientific Instruments, San Francisco, CA) with the nitrocellulose membrane at the anode. After transfer, the nitrocellulose sheet was blocked by 50 ml of 5% nonfat milk in PBS. The nitrocellulose sheet was washed once with PBS and transferred to a plastic bag containing B9 mAb (10 μg/ml) for IEC lysates or 4G10 antiphosphotyrosine mAb (0.5 μg/ml) for T cell–IEC cocultures at 4°C overnight. The sheet was washed five times with PBS for 5 min each, and then transferred to a new plastic bag containing 0.5 μg/ml goat anti–mouse IgG conjugated with horseradish peroxidase (Organon Teknika-Cappel, West Chester, PA). The incubation was performed at room temperature for 2 h. The sheet was then washed five times with washing buffer (0.05% Tween 20 in PBS) for 5 min each, and finally incubated with 12 ml of chemiluminescence reagent (DuPont, Wilmington, DE) at room temperature for 1 min. A sheet of plastic wrap was placed onto the nitrocellulose sheet, and the sheet was exposed and developed (XAR-5 film; Eastman Kodak Co., Rochester, NY).

Results

Expression of gp180 is reduced in IBD. Immunohistochemical staining using anti-gp180 mAbs B9 and L12 revealed striking differences between tissues derived from normal intestine and those from IBD specimens. As seen in Fig. 1 *A*, staining of normal mucosa with mAb B9 revealed the presence of gp180 in both surface and crypt epithelium (surface > crypt), with both an apical and basolateral distribution. At higher magnification, one can see intense apical staining with more evenly distributed basolateral staining (Fig. 1 *B*). Studies from our laboratory have shown that the apical staining in cell lines is reduced after treatment with phosphatidylinositol-specific phospholipase C while the basolateral staining is maintained. These findings suggest that the apically sorted form of gp180 is glycosylphosphatidylinositol-anchored, whereas the basolateral form is likely to be transmembrane (12). The staining of epithelial cells by mAb B9 from normal controls was comparable regardless of the site (cecum; ascending, transverse, descending, or sigmoid colon; rectum; or ileum) from which the IEC were isolated (see below, and data not shown).
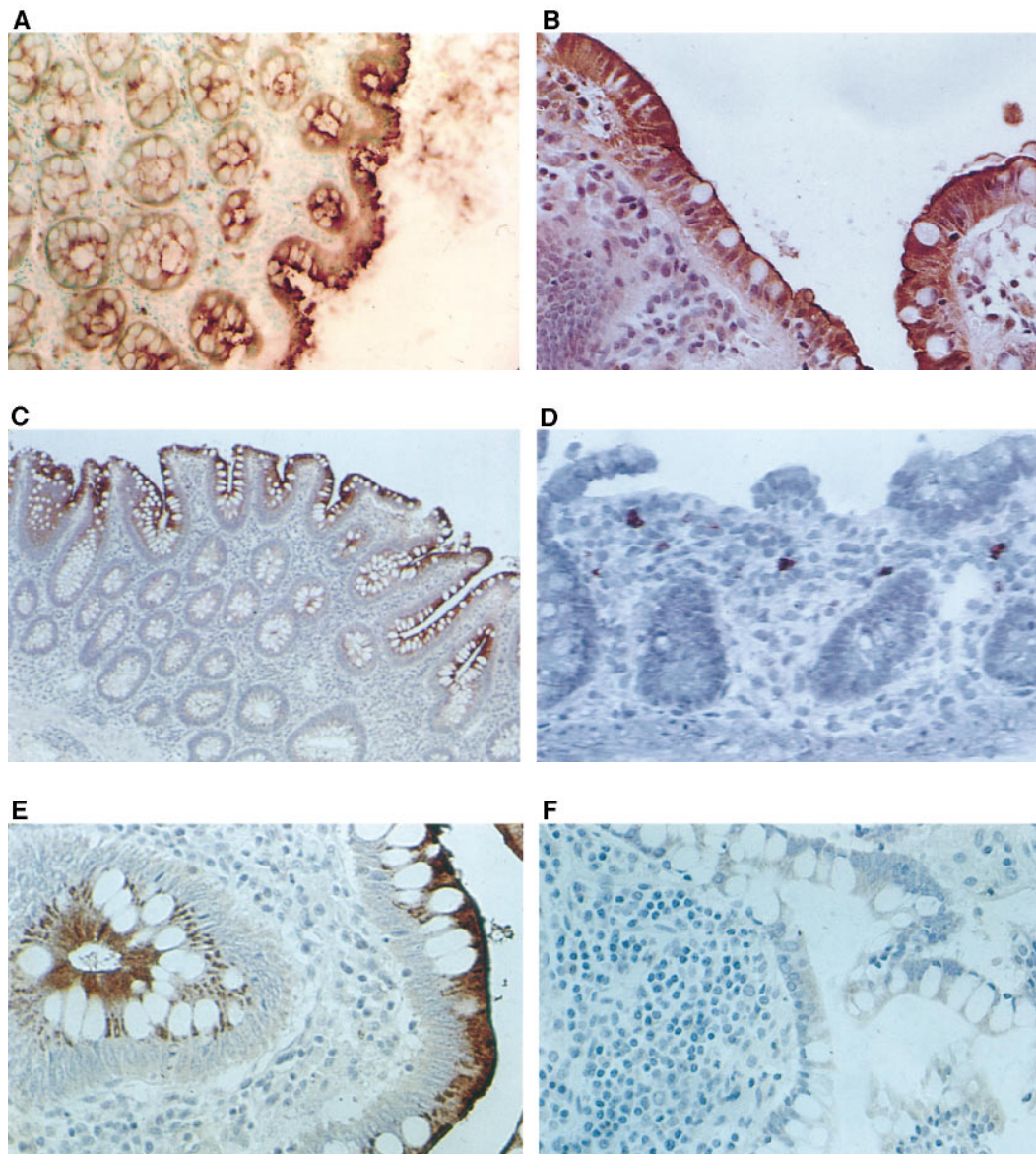

Figure 1. Immunohistochemical staining of frozen sections from (*A*) normal intestinal tissue using anti-gp180 mAb B9 at low magnification (×10) and (*B*) higher magnification (×20); a UC intestinal specimen using anti-gp180 mAb B9, (*C*) (×10) and (*D*) (×20) showing directly adjacent areas, and (*E*), high power magnification (×40) of staining area; and (*F*) an uninflamed region of a CD intestinal specimen using anti-gp180 mAb B9 (×20). Fresh tissue sections were obtained at the time of surgery and snap-frozen immediately in Tissue Tek. Serial 4-μm-thick frozen sections were cut, and indirect staining with mAb B9 was performed using an avidin-biotin-peroxidase technique, with Gill's hematoxylin as a counterstain.
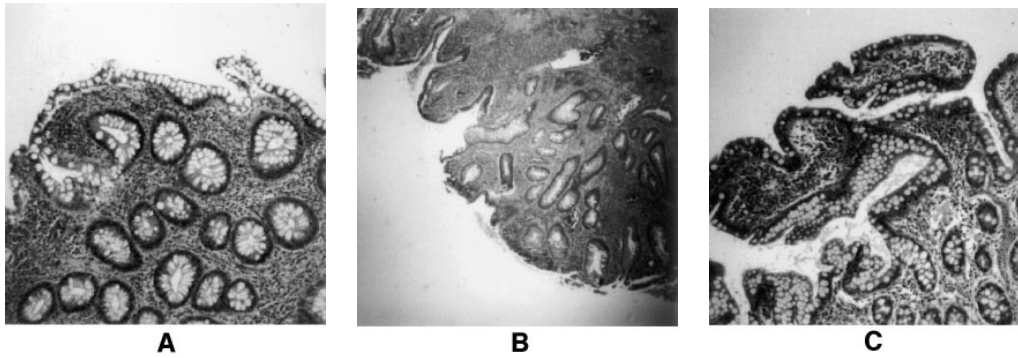
Figure 2. Hematoxylin and eosin staining of frozen sections from IBD intestinal specimens. (*A*) Inflamed (×10) and (*B*) uninflamed (×10) areas of colon from the same UC patient shown in Fig. 1, *C–E.* (*C*) Moderately inflamed (×10) area from the same CD patient shown in Fig. 1 *F.*

In contrast to these findings, staining with both mAbs B9 and L12 in bowel sections derived from patients with UC revealed a more patchy distribution, some areas staining with normal intensity, while other adjacent areas showed no staining at all (Fig. 1, *C* and *D*; hematoxylin and eosin–stained sections from the same patient are seen in Fig. 2, *A* and *B*). However, even in the mAb B9–stained areas, the pattern of staining was altered. There appeared to be a loss of basolateral staining, with preservation or even enhancement of apical gp180 (Fig. 1 *E*). These changes were noted both in areas of active inflammation and in areas where there was no gross or histologic disease (e.g., ileum and right colon). Significantly, this alteration in gp180 expression was observed consistently in all intestinal tissues derived from UC patients, regardless of their history and medical regimen (including patients on no medical therapy at all).

The difference in staining of epithelial cells with mAbs B9 and L12 in patients with CD was even more dramatic. In tissues obtained from both inflamed and uninvolved regions of bowel, little to no staining with either mAb B9 or L12 was noted (Fig. 1 *F*; the immunohistochemically stained section is from an uninflamed region. Hematoxylin and eosin–stained sections from the same patient are seen in Fig. 2 *C*). The marked decrease in staining was seen in all intestinal tissues derived from patients with CD, in inflamed and uninflamed areas. As seen in UC, there was no correlation of staining patterns with any medical regimen. Of note, the pattern of gp180 expression seen in CD (uniformly decreased B9 staining) differs from that observed in UC (patchy and more apical, less basolateral B9 staining). These findings suggest a difference between CD and UC; in the former, a more global deficiency in gp180 expression exists, whereas in UC, there appears to be a reduction as well as an intracellular redistribution of the molecule. Clearly, the uniform defect in CD and the patchy defect seen in UC do not explain the typical tissue distribution of each of these disorders. Other factors must be playing a role in the site of disease expression (see Discussion).

Quantitation of gp180 in normal and IBD IEC. To confirm the immunohistochemical studies in a quantitative manner, isolated IEC stained with mAbs B9 and L12 were analyzed by flow cytometry (Fig. 3 *A*). Normal IEC stained in a uniform distribution, with a fluorescence intensity slightly less than that seen by staining with W6/32, an mAb to class I MHC. IEC derived from inflamed non-IBD tissues (DIV, ischemic colitis) revealed a staining pattern similar to normal controls. However, IEC from UC tissues showed a bimodal pattern of staining: one population with a fluorescence intensity similar to that

seen in normal IEC, and one nonstaining population. This result correlates with the patchy staining seen in the immunohistochemical studies of UC mucosa. The striking decrease in staining in CD IEC observed in immunohistochemical studies was also observed by flow cytometry. IEC from CD demonstrated very little or no staining with mAb B9 or L12. As in the immunohistochemical studies, no correlation between medical regimen and gp180 expression was found. In addition, IEC were gated for selectively by concurrent staining with the anticytokeratin antibody CAM 5.2 (Fig. 3 *B*). Similarly, CD45+ contaminating lymphocytes were gated out in these analyses (data not shown). Thus, the differences in staining reflected differences in the IEC and not in contaminating populations.

Notably, IEC derived from both actively inflamed and uninflamed IBD tissues revealed similar patterns of staining, again suggesting that inflammation in itself is not responsible for the differences seen between normal intestine and IBD specimens. Interestingly, although the overall pattern of staining remained the same, some upregulation of staining with mAb B9 or L12 was observed in the actively inflamed IBD tissues, when compared with uninflamed areas from those same specimens. These findings are consistent with preliminary experiments in our laboratory which demonstrate that gp180 can be upregulated by IFN-γ and GM-CSF, cytokines present in increased amounts in IBD mucosa (20).

When all samples were analyzed together [normal tissue (NL), inflamed and uninflamed UC (UCI and UCU), and inflamed and uninflamed CD (CDI and CDU) IEC groups], clear patterns emerged. Whereas staining of NL control IEC with mAb B9 revealed a wide scatter of fluorescence intensities, the staining intensities of the CDU, CDI, and UCU groups were significantly different from that of the NL group when compared by Student's t test (Fig. 4; NL vs. CDU, $P < 0.003$; NL vs. CDI, $P < 0.01$; and NL vs. UCU, $P < 0.04$). Although not significantly different ($P < 0.14$), the staining by mAb B9 within the UCI group tended to be lower than that seen in the NL group.

The differences in gp180 expression might relate to the possibility that there is a defect in transporting this glycoprotein to the surface in IBD IEC. While this was less likely, given the absence of staining in fixed immunohistochemical sections, we chose to assess this possibility by analyzing total gp180 by mAb B9 Western blot analysis of IEC lysates. The intensity of the 180-kD band in both uninflamed UC and CD IEC was markedly reduced or absent when compared to the band in NL IEC (Fig. 5). Thus, the failure to express gp180 in IBD tissues
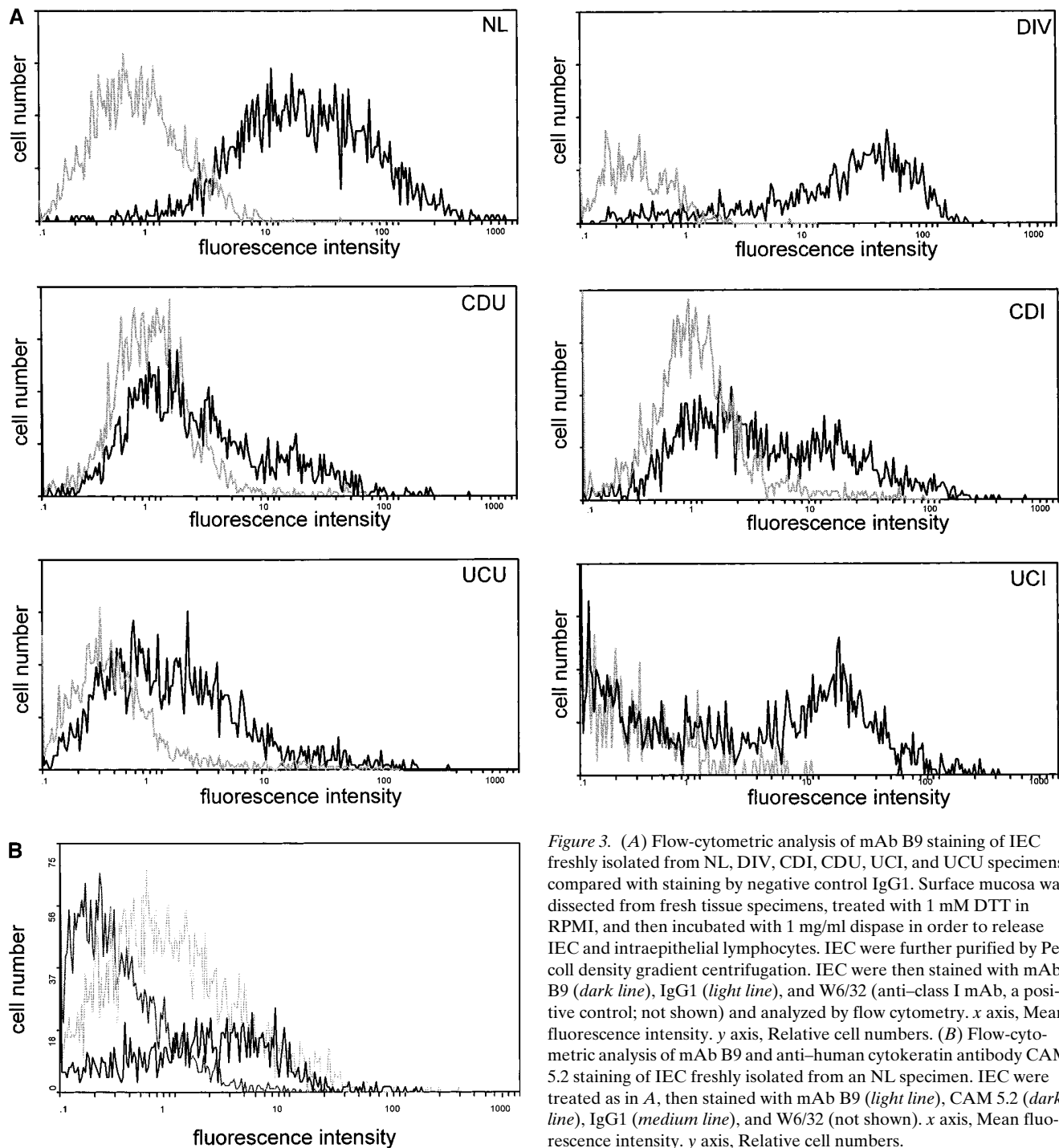
Figure 3. (*A*) Flow-cytometric analysis of mAb B9 staining of IEC freshly isolated from NL, DIV, CDI, CDU, UCI, and UCU specimens, compared with staining by negative control IgG1. Surface mucosa was dissected from fresh tissue specimens, treated with 1 mM DTT in RPMI, and then incubated with 1 mg/ml dispase in order to release IEC and intraepithelial lymphocytes. IEC were further purified by Percoll density gradient centrifugation. IEC were then stained with mAb B9 (*dark line*), IgG1 (*light line*), and W6/32 (anti–class I mAb, a positive control; not shown) and analyzed by flow cytometry. *x* axis, Mean fluorescence intensity. *y* axis, Relative cell numbers. (*B*) Flow-cytometric analysis of mAb B9 and anti–human cytokeratin antibody CAM 5.2 staining of IEC freshly isolated from an NL specimen. IEC were treated as in *A*, then stained with mAb B9 (*light line*), CAM 5.2 (*dark line*), IgG1 (*medium line*), and W6/32 (not shown). *x* axis, Mean fluorescence intensity. *y* axis, Relative cell numbers.

appears to result from reduced production of the form recognized by these mAbs.

Functional consequences of reduced gp180 expression. We next wanted to determine whether this alteration in gp180 expression correlated with a functional phenotype. As described previously (14), the selective stimulation of CD8+ T cells by NL IEC appears to require the activation of a src-like tyrosine kinase, p56*lck*, associated with the intracytoplasmic tail of the CD8α chain. Activation of p56*lck* results in autophosphorylation as well as phosphorylation of associated substrates. In-

hibition of this kinase inhibits CD8+ T cell activation by NL IEC. Furthermore, addition of the anti-gp180 mAb B9 to T cell–IEC cocultures inhibits the activation of p56*lck*, whereas anti–class I and anti–class II mAbs do not. To define more carefully specific pathways involved in T cell–IEC interactions, we used murine T cell hybridomas transfected with either full-length human CD4 cDNA (3G4) or full-length CD8α cDNA (3G8). The only human proteins expressed on these cells are either CD4 or CD8. Others have shown that murine p56*lck* can associate with either of these molecules (18, 19). Cross-
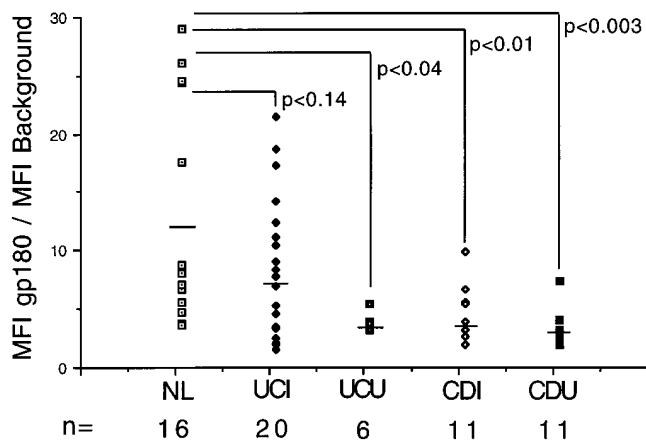
Figure 4. Mean fluorescence intensities of mAb B9 staining in samples of freshly isolated IEC, compared with background, by group. IEC were isolated and treated as described in Fig. 2. Short lines depict the mean values for each group.
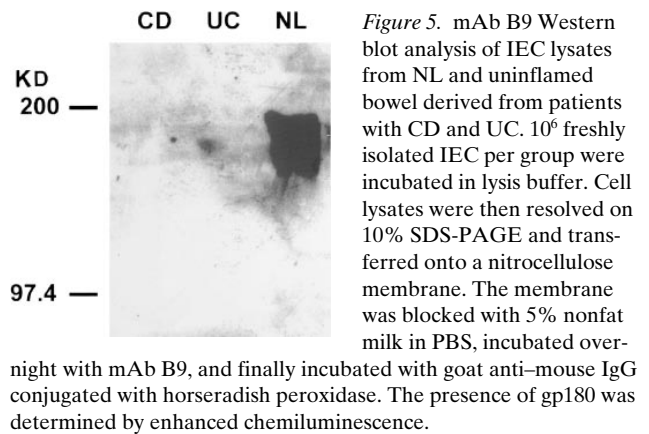

Figure 5. mAb B9 Western blot analysis of IEC lysates from NL and uninflamed bowel derived from patients with CD and UC. 10^6 freshly isolated IEC per group were incubated in lysis buffer. Cell lysates were then resolved on 10% SDS-PAGE and transferred onto a nitrocellulose membrane. The membrane was blocked with 5% nonfat milk in PBS, incubated overnight with mAb B9, and finally incubated with goat anti–mouse IgG conjugated with horseradish peroxidase. The presence of gp180 was determined by enhanced chemiluminescence.

linking of CD4 on 3G4 with anti-CD4 mAbs, or cross-linking CD8 on 3G8 with anti-CD8 mAbs, will result in the autophosphorylation of p56*lck* and associated substrates. Thus, activation of p56*lck* in these transfectants by human IEC can occur only via binding of CD4 or CD8, and each cell line serves as a control for the other.

The results of mixed T cell–IEC coculture assays using 3G4 and 3G8 T cells are shown in Figs. 6–8. As described previously, when NL IEC are cocultured with 3G4 cells, no upregulation of p56*lck* is seen when compared with baseline at 0 min (Fig. 6 A) (13). In contrast, p56*lck* is activated in 3G8–IEC cocultures at 1–5 min, supporting the concept that IEC express a molecule capable of binding to CD8, allowing for kinase activation (Fig. 6 B). In multiple experiments, the increase in CD8-associated p56*lck* was between 3- and 10-fold. Importantly, neither blocking anti–class I nor anti–class II mAbs can inhibit this activation event. Only antibody to gp180 (mAb B9) can inhibit the activation of p56*lck* in this system (13). The in-

crease in the baseline phosphorylation in 3G8 cells appears to reflect the dimerization of CD8α chains but does not affect the response of these cells to CD8 ligation.

Since IEC derived from IBD tissues induce the proliferation of CD4+ T cells but not CD8+ T cells, we asked whether this finding correlated with a different pattern of p56*lck* activation. Freshly isolated IEC from both inflamed and uninflamed areas of the colon from a patient with UC were cocultured with 3G4 and 3G8 cells. In contrast to cocultures with NL IEC, UC IEC enhanced phosphorylation of p56*lck* in 3G4 cells (Fig. 6 A). IEC from both inflamed and uninflamed tissues activated CD4-associated *lck*, although the kinetics of activation were slightly different. Peak activation of CD4-associated p56*lck* by UCU IEC occurred at 10 min, while UCI IEC strongly activated-CD4 associated p56*lck* at 1 min. This correlates with the known increase in class II MHC expression on IEC from inflamed areas. Class II antigen binding to CD4 activates CD4-associated p56*lck*. When 3G8 cells were incubated with UCU IEC, no upregulation of CD8-associated p56*lck* was seen. However, in cocultures with UCI IEC, there was a modest but evident increase in the phosphorylation of CD8-associated *lck* at 1 min (a roughly twofold increase in signal), which was less than that induced by NL IEC. This suggests that CD8 ligation
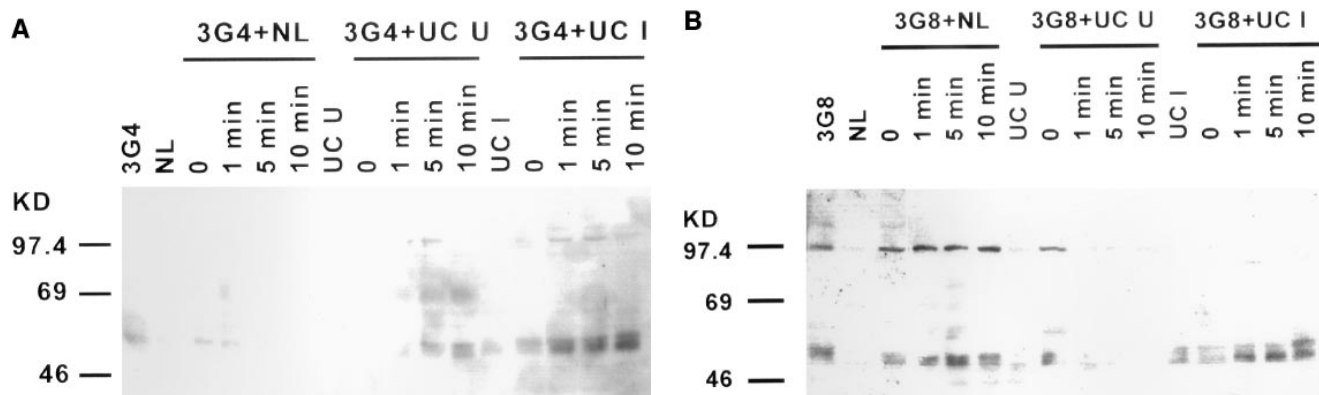

Figure 6. Antiphosphotyrosine Western blot analysis of cell lysates from mixed T cell–IEC cocultures. (A) 3G4 or (B) 3G8 cells were cocultured with NL, UCU, or UCI IEC for 0, 1, 5, or 10 min. Stop buffer was added immediately, and the T cells were lysed with lysis buffer. The lysates were resolved on 10% SDS-PAGE and transferred onto nitrocellulose membranes. The membranes were blocked with 5% nonfat milk in PBS, incubated overnight with 4G10 antiphosphotyrosine mAb, and finally incubated with goat anti–mouse IgG conjugated with horseradish peroxidase. Activation of p56*lck* was determined by enhanced chemiluminescence. Negative control lanes consisting of 3G4 cells, 3G8 cells, and NL, UCU, and UCI IEC alone are also shown.
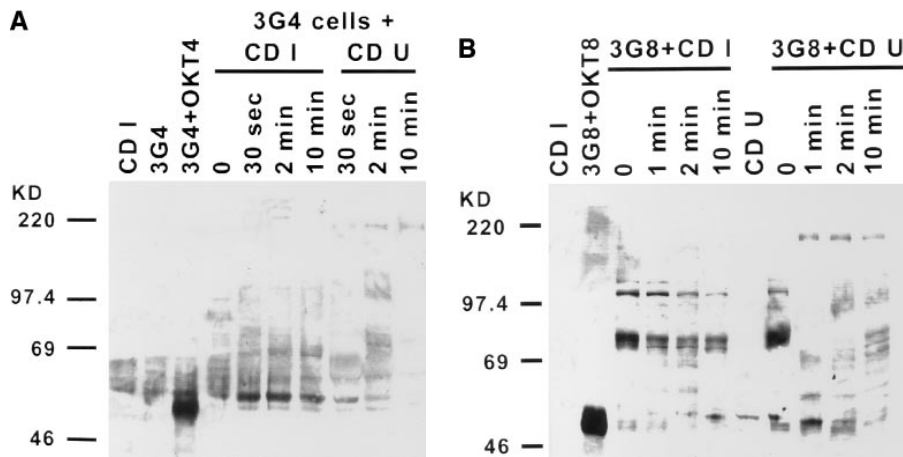
Figure 7. Antiphosphotyrosine Western blot analysis of cell lysates from mixed T cell–IEC cocultures. (*A*) 3G4 or (*B*) 3G8 cells were cocultured with CDU or CDI IEC for 0, 0.5, 1, 2, or 10 min. Cell lysates were prepared, transferred to nitrocellulose membranes, and the membranes were treated as described in Fig. 5. Activation of p56*lck* was determined by enhanced chemiluminescence. Negative controls consisting of 3G4 cells and CDI and CDU IEC alone are also shown. Positive controls showing p56*lck* activation when incubating 3G4 cells with anti-CD4 mAb (*OKT4*) and 3G8 cells with anti-CD8 mAb (*OKT8*) are demonstrated as well.

may be occurring with UC IEC but that the interaction is weaker. These findings correlate with the present but weaker expression of gp180 on UCI IEC (CD8-associated p56*lck* activation) as well as the enhanced expression of MHC class II molecules on these cells (CD4-associated p56*lck* activation).

Like UC IEC, IEC from patients with CD activated p56*lck* in 3G4 cells (Fig. 7 *A*). However, once again, CD4-associated *lck* was activated more by the inflamed than the uninflamed IEC. Maximum activation by CDU and CDI IEC both occurred at 30 s in 3G4–CD IEC cocultures. Cocultures with 3G8 showed limited CD8-associated *lck* upregulation for both CDI and CDU IEC, when compared with baseline (Fig. 7 *B*). These results suggest that CD8+ T cell activation is impaired in CD as well, and may help to explain the previous observations showing a defect in induction of CD8+ T cell proliferation by IEC derived from both CDI and CDU tissues (13). The reduced-to-absent upregulation of CD8-associated p56*lck* in this system correlates with the decrease in gp180 expression by these cells.

Mixed T cell–IEC coculture assays using 3G4 and 3G8 cells were also performed with IEC derived from a non-IBD inflammatory control specimen, DIV (Fig. 8). As in studies using

NL IEC, activation of p56*lck* was seen at 1 min in cocultures with 3G8 cells, but no phosphorylation was observed in the experiments with 3G4 cells. Thus, correlating with B9 staining, differences in p56*lck* phosphorylation patterns between NL and IBD IEC do not appear to be due to the state of inflammation per se.

Discussion

Previous studies from this laboratory have documented a defect in IEC stimulation of T cells in patients with IBD (10). In contrast to NL IEC, which stimulated CD8+ suppressor T cells in allogeneic mixed-cell cultures, IEC derived from both inflamed and uninflamed areas of IBD tissues activated potent CD4+ helper T cells. This difference in CD4+ versus CD8+ T cell activation did not appear to relate to enhanced class II antigen expression by these cells since other non-IBD inflammatory control IEC (ischemic colitis, gold-induced colitis, DIV) were perfectly capable of stimulating CD8+ T cells even in the face of comparable increases in MHC class II antigens. Thus, it appeared that there might be a fundamental defect in
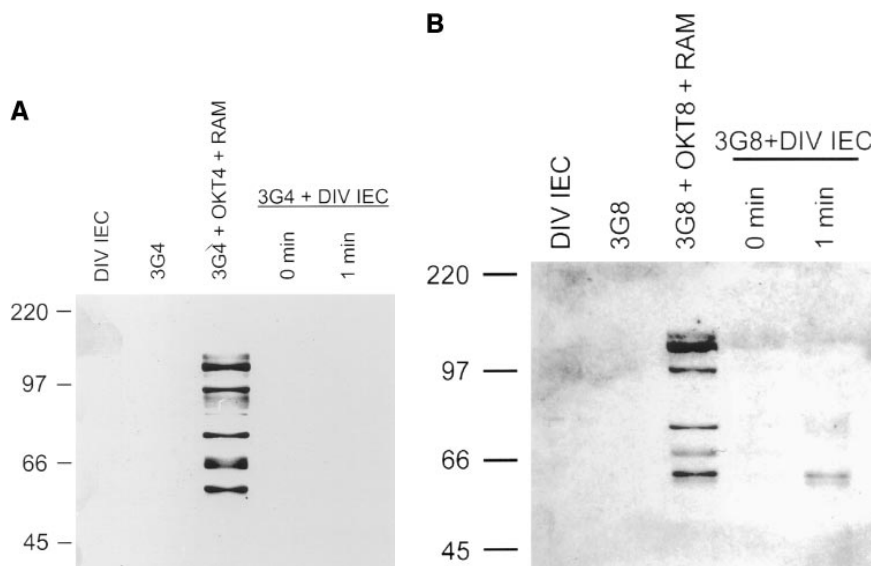

Figure 8. Antiphosphotyrosine Western blot analysis of cell lysates from mixed T cell–IEC cocultures. (*A*) 3G4 or (*B*) 3G8 cells were cocultured with IEC freshly isolated from DIV specimens for 0, 1, 2, or 10 min (last two lanes not shown). Cell lysates were prepared, transferred to nitrocellulose membranes, and the membranes were treated as described in Fig. 6. Activation of p56*lck* was determined by enhanced chemiluminescence. Negative controls consisting of DIV IEC alone, 3G4 cells, and 3G8 cells are also shown. Positive controls showing p56*lck* activation when incubating 3G4 cells with anti-CD4 mAb (*OKT4*) and 3G8 cells with anti-CD8 mAb (*OKT8*) are demonstrated as well.

the ability of IBD IEC to activate suppressor T cells. We postulated that if IEC were functioning as nonprofessional antigen–presenting cells in the gut, antigens processed through IBD IEC would not only result in the failure to shut off mucosal immune responses but also participate actively in the immune activation reported in IBD by a number of groups.

We have identified recently two mAbs, B9 and L12, which inhibit selectively the activation of CD8+ T cells by normal IEC (11). The mAbs B9 and L12 appear to recognize carbohydrate moieties on gp180, a molecule which has the ability to bind to CD8 and activate its associated kinase p56lck, an event essential for T cell activation in this system (12). If gp180 is responsible for CD8+ T cell activation in the normal intestine, we postulated that the defect in CD8+ suppressor T cell activation by IBD IEC (14) might relate to differences in the expression of this molecule. Of note, we found that neither anti–class I nor anti–class II mAbs inhibit IEC-driven CD8+ T cell proliferation, suggesting that classical MHC antigens are not involved (13).

In this study, we demonstrate that mAbs B9 and L12 bind to apical and basolateral sites on surface and crypt IEC from NL and non-IBD inflammatory controls, but minimally stain CD IEC, and bind to UC IEC in a reduced and redistributed pattern. Thus, gp180 expression appears to be defective in IBD. Significantly, the abnormal staining patterns are observed in both inflamed and uninflamed areas of intestine, indicating that inflammation per se is not responsible for the differences seen. Indeed, inflammatory specimens from patients with DIV and ischemic colitis manifest a normal staining pattern. Furthermore, if anything, a reduction in inflammation correlates with a more striking decrease in gp180 expression in IBD tissues, as noted when comparing uninvolved areas with inflamed areas (Fig. 3). In addition, no correlation between medical regimen and gp180 expression was found. Hence, medication does not appear to play a role in the data described here. These findings, taken together with our previous observations, suggest that defects in gp180 expression may be intrinsic to IBD.

It is intriguing to note that the pattern of gp180 expression in the two diseases does not reflect the tissue distribution normally noted in these disorders (patchy expression in UC, where disease is more uniform, and diffusely decreased staining in CD, where disease is more patchy). However, in both cases, the ability of gp180 to interact with T cells is reduced, since apically expressed gp180 is not likely to come in contact with T cells. Thus, both diseases may be functionally similar in terms of gp180–CD8 interactions. The differences in the pattern of disease expression are likely to be due to other factors, such as defects in the mucosal barrier (allowing for greater antigen access to IEC), luminal bacterial content, and underlying genetic predisposition. It has become increasingly apparent that both CD and UC are multigenic disorders. Some genes may be common to both diseases, while others provide the substrate for disease specificity. gp180 expression defects in IBD may have similar functional consequences with different genetic defects. The functional defect gives rise to the ability of IEC to activate CD4+ T cells while failing to simultaneously (or preferentially, as suggested in our DIV studies) activate CD8+ T cells.

In addition, we found that defects in gp180 expression by IBD IEC correlate with alterations in T cell activation by IEC. Our experiments with the transfectant cell lines 3G4 and 3G8 show that while normal IEC activate CD8- but not CD4-asso-

ciated p56lck, IEC derived from both inflamed and uninflamed UC and CD preferentially activate CD4-associated p56lck. CD8α-associated lck and its substrates are also phosphorylated in T cell–IBD IEC cocultures, but to a lesser degree. These findings corroborate several pieces of evidence that strongly support a role for activated CD4+ T cells in the pathogenesis of IBD and the observation that, unlike normal IEC, IBD IEC activate CD4+ T cells but fail to stimulate CD8+ suppressor T cells (9, 10). Given that binding to gp180 appears to be essential to CD8+ suppressor T cell activation in T cell–IEC cocultures [in that antibodies to gp180 inhibit the activation of CD8-associated p56lck in T cell–IEC cocultures (13), and purified gp180 activates CD8+ T cells (12)], these data suggest that defects in gp180 may play a pathogenetic role in the IBD disease process by disrupting CD8 activation, leading to a failure of immune suppression. Activation of CD4+ T cells by IBD IEC can then occur unimpeded.

These findings provide a potential mechanism for the large number of studies documenting an increase in activated CD4+ T cells in IBD mucosa (21–25). These activated CD4+ T cells secrete cytokines that enhance the inflammatory state. Indeed, it has been suggested that the profile of cytokines produced by the CD4+ T cells may dictate the type of disease observed (type 1 for CD, and type 2 for UC) (3). Our data do not provide insights into selective CD4+ T cell activation, although this, too, is likely to result from the genetic predisposition to mount type 1 versus type 2 responses to specific antigens. What our data do suggest is that luminal antigen (and it may be any luminal antigen, but more likely bacterial) sampled by IBD IEC will more likely present these antigens to CD4+ T cells. The nonselectivity of the inducing antigen may help to explain the inability to identify one specific bacterium or virus which is causative in these disorders.

Our work expands on the growing body of literature demonstrating that an interruption of normal T cell homeostasis is critical to the development of IBD. The development of colitis in IL-2, IL-10, and TGF-β knockout mice highlights the importance of cytokine-mediated regulation of T lymphocytes (26–28). Here, too, CD4+ T cells appear to play a significant role in the induction of colitis. Rudolphi and co-workers (29) have shown that intestinal CD4+ T cells from normal mice home preferentially to the intestinal mucosa and trigger disease upon transfer into severe combined immunodeficient (SCID) mice. Intriguingly, Powrie et al. (30–32) and Morrissey et al. (33) have defined specific subpopulations of normal CD4+ T cells, expressing high or low amounts of CD45RB, which have the capacity to induce or prevent colonic inflammatory disease, respectively, upon transfer into SCID mice. Moreover, investigators have discovered large numbers of CD4+ cells expressing low levels of CD45RB and L-selectin in IL-2 knockout mice (30–34). The development of colitis after bone marrow transplantation in tgε26 mice, where thymic development is distorted, is another example of dysregulated CD4+ T cell activity (35, 36). These studies, in addition to the CD45RB^{hi}/SCID transfer model, underscore the critical nature of the absence or presence of regulatory T cells in the induction of disease. Our data support evidence demonstrating a strong role for CD4+ T cells and a disruption of regulatory T cell mechanisms in the pathogenesis of IBD.

The nature of the defect in gp180 expression in IBD IEC is not clear, but the pattern of immunohistochemical staining seen with mAbs B9 and L12 suggests that the defect is post-

transcriptional. In UC IEC, there is a loss of basolateral staining, with preservation of gp180 expression at apical sites, intimating that the alteration is unlikely to be at the protein level, but occurs perhaps during posttranscriptional modification, causing a redistribution of the molecule. The differences in staining may also relate to altered glycosylation of gp180, since both mAbs B9 and L12 recognize N-linked carbohydrate epitopes (12). Further evidence supporting some posttranslational defect in both UC and CD IEC is that gp180 can be upregulated, as shown by our studies demonstrating upregulation in inflamed versus uninflamed IBD IEC and upregulation of gp180 expression by INF-γ and GM-CSF in epithelial cell lines (20). However, the decrease in staining with mAb B9 or L12 on CD IEC at both apical and basolateral sites suggests that the defect in CD is distinct from that found in UC.

In summary, a more global defect in gp180 expression in CD IEC would explain the failure of these cells to activate CD8+ suppressor T cells. For UC IEC, where gp180 expression remains partially intact, the explanation is less clear. However, the absence of normal gp180 expression on UC IEC at basolateral sites, where interactions with lamina propria lymphocytes and intraepithelial lymphocytes are most likely to occur in vivo, may be one mechanism by which UC IEC fail to activate suppressor cells in the host. Regardless, these findings may help to explain the uncontrolled inflammation seen in IBD. In this model, presumably any luminal antigen might trigger the initial immune response through the activation of CD4+ T cells, and the failure of gp180 to activate CD8+ T cells explains its perpetuation.

Acknowledgments

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