T cell receptor δ repertoire in inflamed and noninflamed colon of patients with IBD analyzed by CDR3 spectratyping

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INFLAMMATORY BOWEL DISEASE (IBD) comprises at least two human diseases of unknown etiology: Crohn’s disease (CD) and ulcerative colitis (UC) (5). CD is characterized by a transmural inflammation and by skip lesions with macroscopically normal intervening mucosa among disease areas. The preferred localization of the disease is the terminal ileum, but the disease may affect any part of the gastrointestinal tract. One hallmark of CD is the granuloma formation in mucosal lesions. In contrast, the inflammatory lesions in UC are confined to the epithelium and the mucosa of the colon. Evidence that the immune system plays a major part in the pathophysiology of both diseases (5) derives from the ability to suppress these diseases by immunosuppressive therapy and from genetically modified animals with altered regulation of the immune system that develop severe chronic intestinal inflammation (15, 41). Initial cellular events that take place at the outset of IBD are poorly understood. CD and UC resemble autoimmune diseases, and numerous publications support the notion that T cell-mediated mechanisms may play a pivotal role in the pathogenesis of the disease (41). For example, there is an increased number of activated Lamina propria lymphocytes in the inflamed mucosa of patients with CD (7, 11). These mucosal T cells might have lost the physiological hyporesponsiveness to enteric antigens (14). An important role of T cells is also supported by various animal models of IBD, showing that gut inflammation can be induced and transferred by T cells (36, 38, 42). In addition, normal microflora is necessary in the development of gut inflammation in all animal models (15), indicating that microbial antigens might activate autoreactive T cells. Thus T cells that specifically accumulate in mucosal lesions may represent a primary oligoclonal T cell response that triggers the onset of the disease. Clonally expanded α/β T cells were described in the inflamed intestine of patients with CD and UC (9, 39, 43, 48, 49), and similar expansions could be also found in the peripheral blood (44). Although many studies have suggested a pathogenic role for α/β T cells, little work has addressed γδ T cells.

γδ T cells are a minor T cell population in humans, and their functions are still largely unknown (reviewed in Refs. 25 and 33). It was suggested that they play an important role in regulating the mucosal immune response and are key mediators of autoimmune diseases (26). Intraepithelial γδ T cells are thought to produce keratinocyte growth factor, which suggests a role in...
maintaining the integrity of the epithelium (3). However, two recent studies (16, 47) suggest that this might not be the case. We have previously shown that the human small and large intestine of healthy adults is populated by clonally expanded γδ T cells that are widely distributed (10, 28, 31). Furthermore, the human skin, which forms another large surface to the external environment, is also composed of clonally expanded γδ T cells that are widely distributed (10, 28, 31). Furthermore, the human small and large intestine of healthy adults is thought to respond to self antigens rather than to foreign antigens (1, 22, 24).

Several studies support the notion that γδ T cells play a key role in the pathogenesis of IBD. Immuno-histochemical studies (37) reported an increased number of γδ T cells in the inflamed mucosa, although contradictory studies (6, 12, 53) were published as well. Furthermore, γδ T cells were found to be frequent in T cell areas around lymphoid follicles and epithelioid granulomas of CD (19), and clonal γδ T cell expansions were described in the inflamed mucosa of patients with CD (35). In addition, analysis of peripheral blood lymphocytes revealed an increased percentage of γδ T cells expressing the T cell receptor DV1 (TCRDV1) gene segment (6, 20, 51). In normal subjects, TCRDV2 is preferentially expressed by peripheral γδ T cells (4), whereas intestinal γδ T cells express mainly TCRDV1 (13, 28). TCRDV1 is also the dominant V region in the inflamed mucosa of patients with IBD (37, 51). Thus circulating TCRDV1-expressing γδ T cells might be derived from colonic γδ T cells leaving the inflamed gut.

The significance of these findings in subjects with IBD is unclear, because it is not known whether the same expanded γδ T cell clones are also present in the noninflamed intestinal tissue or the peripheral blood. The finding of clonality within the inflamed colon does not allow the conclusion that they are disease related, because healthy subjects already express a highly oligoclonal TCR-δ repertoire (10, 28, 31). However, it might be possible to identify autoreactive γδ T cell clones specifically expanded only within IBD lesions by comparing them with γδ T cells from noninflamed mucosa. Because T cell clones are generally activated and clonally expanded through contact of their clonotypical TCR with an appropriate antigen, we evaluated the complementarity determining region 3 (CDR3) regions of TCR-δ transcripts of mucosal and peripheral lymphocytes from patients with CD and UC and compared them with those of inflammatory and healthy controls.

**METHODS**

Sample collection and RT-PCR of TCR-δ transcripts. Mucosal biopsies from inflamed and noninflamed mucosa, 2–3 mm in size, were obtained by endoscopy from eight subjects with active CD (F–M) and three subjects with active UC (N–P). From subject L, we obtained additional colon biopsies 5 mo before the exacerbation of CD. From subjects O and P, additional biopsies were available from moderately inflamed mucosa located between the noninflamed and highly inflamed colon. Biopsy specimens from IBD subjects were categorized into those from inflamed and noninflamed intestine according to endoscopic appearances and histology of adjacent samples. In addition, two inflammatory controls with diverticulitis (Q and R) and five normal controls (A–E) were included in the study. The distance between noninflamed and inflamed specimens was ~10–20 cm. From four healthy subjects (A–D), we (29) previously reported about the IgA and IgM variable heavy chain repertoire. The distance between the two colonic biopsies of healthy adults were 1 m (A–C) or 10 cm (D and E). Colonic biopsies were snap frozen immediately in liquid nitrogen. Peripheral blood specimens were obtained at the time of endoscopy, and peripheral blood mononuclear cells (PBMC) were isolated using a Ficoll density gradient (28, 31). The age range of studied subjects was 25–60 yr. All studies were approved by the University of Frankfurt ethical committee on human subjects.

On average, we obtained 7–15 μg of RNA from each biopsy; 1–2 μg were reverse transcribed into cDNA in a 20-μl reaction mix. PCR was performed with 2 μl of this reaction mix. Thus ~100 ng of cDNA was utilized in each PCR reaction. TCRDV1, -DV2, and -DV3 transcripts were amplified with Taq polymerase using V-δ- and C-specific primers as previously described (28, 30, 31). After an initial hot start, amplification of TCRD rearrangements consisted of 37 cycles of 40 s at 94°C, 50 s at 61°C, and 1 min at 72°C, followed by a final extension for 10 min at 72°C. The expected PCR product length was 150–250 bp.

The primers used in this study were TCRDV1: CAG CCT TAC CCT AGC TAG AAG ATT CAG C; TCRDV2: GCA CCA TCA GAG AGA GAT GAA GGG; TCRDV3: TCA CTT GGT GAT CTC TCC AGT GAT G; and TCRDC: AAA CGG ATG GTT TGG TAT GAG GC.

CDR3 spectratyping. For analysis of CDR3 lengths, 2–3 μl of each PCR mixture was added to formamide-containing loading buffer. PCR products were heat denatured for 2 min at 95°C. PCR products were then size separated on a 6% denaturing polyacrylamide gel and visualized by silver staining (Silver Sequence DNA staining reagents) as recommended by the manufacturer (Promega, Madison, WI). Bands were photographed by exposing polyacrylamide gels for 8–15 s to an automatic processor-compatible film (Silver Sequence). Demonstrating the reproducibility of our method, the CDR3 profiles of the two corresponding PBMC samples (Fig. 1, subjects A–D), which were analyzed independently, were almost 100% identical.

Direct sequencing of individual CDR3 length bands. For direct sequencing of TCR-δ rearrangements, individual dominant bands were excised from the gels and incubated at room temperature in 50 μl sterile distilled H2O, after which 5–μl aliquots were reamplified for 30–35 cycles using the same primers and PCR conditions described above. Double-stranded PCR products were directly sequenced using the ABI automatic sequencer 310 and the ABI prism dye terminator cycle sequencing ready reaction kit with AmpliTaq DNA Polymerase, FS (Perkin-Elmer, Weiterstadt, Germany), according to the conditions recommended by the manufacturer. For sequencing, the 3’ primer, which was used for the PCR amplification, was taken.

Sequence analysis and calculation of CDR3 lengths. Nucleotide sequences were analyzed using PC/Genie and OMIGA software (Oxford Molecular, Cambridge, UK). The
lengths of the CDR3 regions of translated TCR-δ chains were calculated as previously described (28, 31, 45). This calculation is arbitrary, because the exact borders of the CDR3 region are not defined and other groups use different calculations. Here, CDR3 lengths were determined by the number of amino acids between the conserved cysteine C, which is encoded near the 3'-end of TCRDV regions, and the conserved GXG triplet, which is encoded by all TCRDJ regions, and

![Fig. 1. Complementarity determining region 3 (CDR3) length profiles of T cell receptor (TCR-δ) transcripts from the colon and peripheral blood of healthy adults. PCR-amplified TCR-δ transcripts from 5 healthy adults (A–E) were size separated on a denaturing gel. Both biopsies (colon I and II) and the two blood samples (peripheral blood mononuclear cells; PBMC I and II) were obtained at the same time. As shown, the overall CDR3 profiles from colon I and II (up to 1 m apart) were highly similar but not 100% identical, indicating that some variation exists among different colonic sites. Dominant bands not present in both colon sites (I and II) are indicated by an arrow. The peripheral TCR-δ repertoire was also oligoclonal but distinct from the colon.](image)
eight amino acids were subtracted (45) [e.g., the CDR3 region: CALGEQRNPLVNWTAQLFFGQG, which is 22 amino acids long, has a CDR3 length of 14 amino acids (22 – 8 = 14)].

RESULTS

CDR3 length analysis of TCR-δ transcripts from the colon and PBMC of healthy subjects. In a first set of experiments, we analyzed the TCR-δ repertoire of the colonic mucosa and PBMC of five healthy adults (subjects A–E). As shown in Fig. 1, the TCRDV1, -DV2, and -DV3 repertoire in normal colon was oligoclonal, and highly similar CDR3 profiles with identical dominant bands were present at distant colon sites separated by a distance as far as 1 m. These findings indicate that dominant γ/δ T cells are widely distributed throughout the colon. However, we occasionally observed expanded γ/δ T cell clones, which were not present at both colon

Fig. 2. CDR3 length profiles of TCR-δ transcripts from the colon and peripheral blood of patients with active Crohn’s disease (see also legend of Fig. 1). Similar CDR3 profiles of TCRDV1 and -DV2 transcripts were present in noninflamed (−) and inflamed (+) colonic mucosa of 6 subjects (F–K). CDR3 profiles of the PBMC samples were distinct from the colonic samples. No PBMC samples were available from subjects J and K. Dominant bands, containing TCR-δ transcripts from clonally expanded γ/δ T cells, were excised from the gels and directly sequenced (Fig. 3). Those bands are indicated by the CDR3 length numbers at the side of the gels.
sites. For example, colon biopsy I of subject A had additional dominant TCRDV1 CDR3 length bands not present in the colon of biopsy II (see arrows in Fig. 1). These minor variations could not be assessed by our previous work where we characterized the TCR-δ repertoire by cloning and sequencing (10, 28, 31). Dominant bands were also present in the peripheral blood. However, the CDR3 profiles of the intestine and the peripheral blood were distinct from each other, indicating that different γδ T cell subsets are present in each organ.

CDR3 length analysis of TCR-δ transcripts from the colon of subjects with CD. TCRDV1- and -DV2-specific CDR3 spectratyping from noninflamed and inflamed colonic mucosa of six subjects with active CD (P-K) revealed oligoclonal γδ T cell expansions (Fig. 2). Minor differences between the CDR3 profiles of noninflamed and inflamed mucosa were within the range seen in healthy subjects when biopsies from different colonic sites were compared (Fig. 1). In contrast, the CDR3 profiles of inflamed and noninflamed mucosa were different in all but one patient (subject G). Because TCRDV3-specific PCR yields were very low in subjects P, I, and J, it is likely that TCRDV3 was rarely expressed. This is in accordance with antibody studies in patients with IBD that demonstrated that TCRDV1 and -DV2 are the major V regions expressed by γδ T cells in the inflamed and noninflamed mucosa (37, 51). Thus it is possible that TCRDV3 transcripts were just above the detection level, which might have skewed the CDR3 profiles. This is supported by our data from subject J, where we obtained different CDR3 profiles from the two noninvolved biopsies. In each of those cases, PCR was repeated with a 10-fold higher amount of cDNA that did not result in stronger PCR products. However, in subjects H and K, good PCR yields were obtained and additional dominant bands were visible in the inflamed mucosa. Thus on the basis of the small number of patients analyzed, we do not know whether those differences are specific for Crohn’s disease or are within the range seen in our healthy controls.

Identical TCR-δ transcripts are present in inflamed and noninflamed mucosa. To confirm that identical clonal expansions were present in inflamed and noninflamed tissue, dominant bands of identical CDR3 length from TCRDV1, -DV2, and -DV3 transcripts were isolated from the gels, reamplified, and directly sequenced. Sequence analysis confirmed identical TCR-δ rearrangements at both colon sites in 18 cases (Fig. 3). In subject K, different TCRDV3 transcripts with a CDR3 length of nine were isolated from involved and noninvolved mucosa.

There is no evidence for “gut-like” γδ T cells in the peripheral blood of subjects with Crohn’s disease. From four patients (P-I) PBMC samples were obtained at the time of endoscopy. Similar to healthy controls (Fig. 1), most CDR3 profiles of peripheral TCR-δ transcripts were also restricted and clearly distinct from those of the intestine (Fig. 2). Thus dominant γδ T cells are unlikely to be derived from the inflamed mucosa as suggested previously (6, 20, 51). From the PBMC sample of subject H, the dominant TCRDV3 band with a CDR3 length of nine was also analyzed, because it was identical in length with the dominant bands of the

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**Fig. 3.** TCR-δ nucleotide sequences of γδ T cell clones expanded in the colon of patients with Crohn’s disease. Corresponding pairs of dominant bands with an identical CDR3 length from noninflamed (−) and inflamed colon (+) of CD patients were excised from the gels (Fig. 2) and directly sequenced without cloning. The two “x” in the fourth column indicate that the same TCR-δ sequence could be isolated from both colon sites. For example, the first sequence FS153 with a CDR3 length of 20 was derived from the noninflamed (−) and inflamed (+) colon of subject F. P nucleotides are underlined. All sequences except JS782 from subject J were in frame. These sequence data are available from EMBL/GenBank/DDJB under accession numbers AF451695–AF451717.
colon. However, sequence analysis revealed that it contained a distinct TCR-δ rearrangement (sequence H5200; Fig. 3), confirming that the repertoires of the blood and the inflamed intestine are different. Similar data were obtained from the patients with UC (see below).

The colonic and peripheral TCR-δ repertoire can be highly diverse. In two subjects with CD (L and M), we identified highly polyclonal CDR3 profiles of mucosal TCRDV1 transcripts (Fig. 4). From one of them (L), we were able to obtain tissue and PBMC during remission and at the onset of active disease 5 mo later. However, there was no significant change in the CDR3 profile over time and the repertoire remained polyclonal. Thus the TCR-δ repertoire can be very variable in CD, but we rarely identified dominant clones expanded only within the inflamed intestine.

Distinct TCR-δ repertoires are present in the highly inflamed colon of subjects with UC. In subject N, the colonic CDR3 profile was polyclonal for TCRDV1 and -DV2 and oligoconal for TCRDV3 (Fig. 5). We do note that the noninflamed colon contained clonal TCRDV1 expansions not visible in the polyclonal repertoire of the inflamed colon. The CDR3 profiles of peripheral TCRDV2 transcripts were polyclonal, whereas peripheral TCRDV1 and -DV3 repertoires were highly oligoconal and distinct from the intestinal TCR-δ repertoire. In the second patient (O) we observed significant changes between normal and diseased colon. The CDR3 profiles of TCRDV1, -DV2, and -DV3 transcripts from the noninflamed and moderately inflamed tissue were highly oligoconal and almost identical. Sequence analysis of dominant bands confirmed identical TCR-δ transcripts at different colon sites (Fig. 6A). However, this typical CDR3 pattern was lost in the highly inflamed areas and a polyclonal repertoire predominated. Possibly this could reflect the influx of peripheral γδ T cells in the inflamed colon, because the TCRDV1 and -DV2 repertoires of PBMC were highly polyclonal as well. In contrast, there was a loss of dominant clones in the third, patient P, when the TCRDV1, -DV2, and -DV3 repertoires of highly inflamed and noninflamed mucosa were compared. Furthermore, sequence analysis of dominant TCRDV3 bands with a CDR3 length of 16 demonstrated that different TCRDV3 rearrangements were present in the inflamed and noninflamed colon (Fig. 6A).

Identical dominant TCR-δ rearrangements are present in inflamed and noninflamed mucosa of patients with diverticulitis. We further studied the TCRDV1, -DV2, and -DV3 profiles of two inflammatory controls (Q and R) suffering from diverticulitis (Fig. 7). Similar to what we observed in the subjects with CD, we did not see a significant change in the CDR3 profiles of TCRDV1/transcripts between inflamed and noninflamed mucosa, and sequence analysis confirmed identical TCRDV1 transcripts at both sites (Fig. 6B). The TCR-δ repertoires of the peripheral blood and the intestine were distinct. The PCR yield of colonic TCRDV3 transcripts of subject Q was low, and we were unable to amplify any TCRDV3 transcripts from the noninflamed mucosa.

Characteristics of mucosal TCR-δ transcripts from subjects with CD, UC, and diverticulitis. All sequences were highly complex, as shown by extensive trimming of the gene segments and multiple N region additions (Figs. 3 and 6). An overutilization of the TCRDJ3 gene segment was suggested before for TCRDV1 and -DV3 transcripts of the inflamed colon of subjects with CD (35). However, none of our TCRDV1 and -DV3 transcripts contained the DJ3 gene segment and the vast majority used DJ1. DJ3 was only found in conjunction with TCRDV2 transcripts. DJ3 is also frequently used by TCRDV2 transcripts in healthy subjects (28). Translation into corresponding amino acid sequences did not reveal any obvious motif shared among different subjects (Fig. 8).

**DISCUSSION**

Previous studies proposed an important role for γδ T cells in the pathogenesis of IBD (6, 20, 51), and an increased number of γδ T cells (19, 37) with clonal expansions was reported (35) in the inflamed mucosa. It was hypothesized that unidentified antigens, presumably from the intestinal microflora, might activate autoreactive mucosal γδ T cells that initiate a destructive immune response. Autoreactive γδ T cells were also thought to be responsible for other autoimmune diseases like multiple sclerosis where several groups...
(32, 50) identified oligoclonal expansions in brain lesions. Thus the identification of clonally expanded γδT cells in the inflamed intestine of subjects with IBD, as suggested before for α/β T cells (9, 23, 39, 43, 48, 49), would support the notion that autoreactive γδ T cells might damage the mucosa.

As shown herein, we rarely identified that γδ T cell clones exclusively expanded only in the inflamed mu-
cosa, and highly similar CDR3 profiles and identical TCR-δ transcripts were present in the inflamed and noninflamed mucosa of most patients. Because the TCR-δ repertoire can also differ between two colonic sites of healthy subjects, we do not know whether those expansions, which were especially found within the TCRDV3 repertoire, are specific for IBD. However, we observed significant changes, such as the diversification of the TCR-δ repertoire in the “loss” of dominant clones, in a subset of patients. A diverse intestinal TCRDV2 repertoire was also described by others in patients with CD. (34). Whereas the diversification of the mucosal repertoire might have been caused by peripheral γδ T cells infiltrating the inflamed mucosa, it is not clear why dominant clones, which are present in the noninvolved mucosa, are absent in the inflamed mucosa of other patients. Thus changes of the TCR-δ repertoire can be highly variable and are distinct from those described for α/β T cells.

Lack of clonally expanded γδ T cells, which are specific for the inflamed mucosa, does not exclude the possibility that γδ T cells play a significant role in the destructive nature of the immune response. First, γδ T cells might be activated by stress-induced self antigens (22) that could lead into tissue damage. It is possible that the γδ TCR repertoire in normal mucosa is already shaped by self antigens under physiological conditions, and further activation through the same self antigens would only cause a proliferation of local γδ T cells with no change of the TCR-δ repertoire. This is supported by our observation that we could not see any change in the repertoire between the inflamed and noninflamed mucosa of subjects with diverticulitis. Second, mucosal γδ T cells might be just bystanders of an ongoing inflammation and their response could be triggered by CD3-independent mechanisms or inflammatory signals such as chemokines alone, which are secreted by other immune cells (46). Third, there might be only a few disease-specific autoreactive γδ T cells within the inflamed mucosa that are very rare but control the vast majority of nonspecific cells (52). Finally, the possibility remains that pathogenic T cell clones are not restricted to inflamed areas but are present throughout the intestine.

Several groups independently described an increase of TCRDV1-expressing γδ T cells in the peripheral blood of subjects with active IBD. Because TCRDV1 is predominantly expressed not only in healthy mucosa (13, 28, 40) but also in the diseased mucosa (37, 51), it was suggested that peripheral γδ T cells are likely to be derived from the inflamed intestine (6, 20, 51). However, this scenario is unlikely, because our data demonstrate that γδ T cell clones, which predominate in the inflamed intestine, are different from those that predominate in the peripheral blood.

In summary, we have shown that there can be significant differences between the TCR-δ repertoire of the inflamed and noninflamed colon, indicating that γδ T cells play a significant role in a subset of patients with IBD. The opposite findings, like the diversification of the TCR-δ repertoire or the loss of dominant clones, might be explained by the hypothesis that CD and UC are not just two diseases but are likely to consist of several different subgroups that all have a distinct pathophysiology (21). In animal...
models, different alterations of the immune system all resulted in a common final pathway: the inflamed intestine (15). Because our findings are distinct from those described for α/β T cells, it is possible that γ/δ T cells might not damage the mucosa but have important secondary roles in IBD like the downregulation of the inflammatory process or regeneration of the damaged mucosa (3). This is supported by studies that suggest that γ/δ T cells induce oral tolerance (26), maintain T cell hyporesponsiveness of mucosal T cells (2, 18), and secrete cytokines promoting growth and differentiation of epithelial cells (3, 17). In addition, γ/δ T cells were shown to have a protective role in rat 2,4,6-trinitrobenzene sulphonic acid colitis, because depletion caused increased mortality (27).

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**Fig. 7.** CDR3 length profiles of TCR-δ transcripts from the colon and peripheral blood of patients with diverticulitis. The CDR3 profiles of TCRDV1, -DV2, and -DV3 transcripts from subjects Q and R were highly similar in noninflamed (−) and inflamed (+) mucosa. Sequence analysis confirmed identical TCRDV1 transcripts at both colon sites (Fig. 6).

**Fig. 8.** TCR-δ amino acid sequences from patients with Crohn’s disease, UC, and diverticulitis. Amino acid sequences of translated TCR-δ transcripts from γ/δ T cell clones expanded in the colon of subjects with Crohn’s disease (Fig. 3) (A), UC (Fig. 6A) (B), and diverticulitis (Fig. 6B) (C).
REFERENCES


