Analysis of Human Common Bile Duct-Associated T Cells

Evidence for Oligoclonality, T Cell Clonal Persistence, and Epithelial Cell Recognition

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The phenotype of T cells associated with the common bile duct (CBD) is unknown. We investigated the hypothesis that they behave like other intraepithelial lymphocytes (IEL). Thus, we sought to determine the phenotype, TCR repertoire, and epithelial recognition of T cells obtained during endoscopic retrograde cholangiopancreatography. Three subjects were studied: two with primary sclerosing cholangitis and one normal control. After establishing a short-term T cell line, cells were 1) stained with mAbs for flow cytometric analysis, 2) analyzed for TCRB chain transcript expression, and 3) used as effector cells for cytotoxicity and proliferation. Flow cytometry revealed that for all the subjects 98% of the T cells were TCR-$\alpha\beta$-positive. Immunohistology of the CBD showed that the epithelium and lamina propria contained significant numbers of $CD3^+CD43^+CD45RO^+$ lymphocytes. Complementarity-determining region 3 length displays suggested that the CBD-derived lines were oligoclonal. This was confirmed by cloning and random sequencing of PCR amplification products using TCRBV region family-specific primers; TCRB chain sequences were reiterated in all transcripts analyzed. In one case, two expanded TCRB clones could be identified that were persistent in the bile duct over a 1-yr period. The CBD-derived lines were cytolytic in a redirected lysis assay and caused cytolysis of an intestinal epithelial cell line (Caco-2). This recognition was likely preferential for intestinal epithelial cells, since a CBD-derived line exhibited proliferation to two intestinal epithelial cell lines (HT-29 and Caco-2) but not three other lines (HepG2, human foreskin fibroblast, and KD). We conclude that the CBD contains IELs that share several characteristics with intestinal IELs. *The Journal of Immunology,* **1997, 158: 1941–1948.**

Most epithelial surfaces that line the mucosa contain a unique population of intraepithelial lymphocytes (IEL),[4] which are predominantly T cells. Although their function remains largely unknown, IELs are commonly believed to play important roles in local immunoregulation and/or immunosurveillance. Under normal circumstances, these functions are likely to be important in the regulation of Ag responsiveness, maintenance of epithelial cell integrity, and protection from invading foreign pathogens. Similarly, abnormalities in these putative functions may underlie the pathogenesis of a variety of diseases possibly of epithelial origin.

In humans, the largest and best studied IEL population is that which is contained within the gastrointestinal tract and is a potential paradigm for other IEL populations. Both human small and large intestinal IELs consist predominantly of $CD45RO^+$, TCR-$\alpha\beta^+$ T cells (1–3) and, to a lesser extent, $\gamma\delta^+$ T cells (4), which bear a unique mucosal integrin, $\alpha^E\beta_7$ (5). Although $CD8^+$ T cells predominate in both sites, $CD4^+$ and double-negative ($CD4^-$/$CD8^-$) T cells may represent a significant fraction of large intestinal IELs (6). Both small and large intestinal IELs also express a limited array of $\alpha\beta^+$ (7–10) and $\gamma\delta^+$ TCRs (11), which, together with their CD45RO expression, indicates that IELs are memory cells directed at a restricted number of Ags regardless of the restriction element on the APC, presumably the intestinal epithelial cell.

The likely focus of these cells is a limited range of Ags, which, together with observed functional consequences of Ag/MHC ligation of the TCR/CD3 complex of human IELs, supports their likely role in immunoregulation and/or immunosurveillance of intestinal epithelial cell function. Human intestinal IELs secrete a variety of potentially immunoregulatory cytokines, such as γ-IFN, that are capable of directly regulating intestinal epithelial cell function (12). Furthermore, human intestinal IELs effect target cell cytolysis, which may be more active against target cells of epithelial origin (13).

Although IELs have also been observed in the epithelium of the bile duct, their phenotype and potential function have received little attention (14–18). Of specific interest, the number of biliary epithelium-associated IELs (bIELs) are increased in diseases such

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[4] Abbreviations used in this paper: IEL, intraepithelial lymphocytes; bIELs, biliary epithelium-associated intraepithelial lymphocytes; CBD, common bile duct; CDR3, complementarity-determining region 3; dNTPs, deoxyribosylnucleotide triphosphate; ERCP, endoscopic retrograde cholangiopancreatography; PBT, peripheral blood T cell; PHA-P, phytohemagluttinin-P; PSC, primary sclerosing cholangitis; TCRB, T cell receptor β-chain; UC, ulcerative colitis; KD, lip fibroblast cell line.

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as primary biliary cirrhosis and primary sclerosing cholangitis (PSC) (14–18). This suggests a potential role of bIELs in the immunopathogenesis of these diseases. However, a major problem in studying human bIELs is their general inaccessibility and relatively low number. To address the structure and function of T cells, we have established an approach that entails the in vitro expansion of bIELs obtained directly from the common bile duct (CBD) at endoscopic retrograde cholangio-pancreaticography (ERCP). This approach is similar to bronchoalveolar lavage in the analysis of pulmonary lymphocytes (19). We show that T cells obtained from CBDs share several critical functional and phenotypic similarities with intestinal IELs including predominant oligoclonal TCR-$\alpha\beta^+$ phenotype and cytotoxicty against epithelial cell lines. Furthermore, we have shown that some individual TCR-$\alpha\beta^+$ clones from the CBD may be persistent over at least 1 yr, implicating a role for specific T cell clones in this site.

Materials and Methods

Subjects and samples

Four brush cytology samples were obtained during the course of therapeutic and diagnostic ERCP from three subjects. Sample 1 was obtained from a subject with primary sclerosing cholangitis (PSC-1) who underwent stent replacement. Sample 2 was from a subject who had PSC as a complication of ulcerative colitis (PSC/UC) and had a stent inserted. Sample 3 was from a normal subject who underwent a diagnostic ERCP for an apparent stricture at the Ampulla of Vater, which was normal. In one case, a second sample (PSC-2) was available 1 yr after isolation of the first sample.

Isolation of T cells and establishing T cell lines

The cytology brush was cut from its wire, placed in RPMI 1640 (Mediatech, Herndon, VA) supplemented with L-glutamine, nonessential amino acids (BioWhittaker, Walkersville, MD), penicillin and streptomycin (100 U/ml; BioWhittaker), and agitated vigorously for 3 min. The medium, still containing the brush, was centrifuged at 300 × *g* for 15 min. The cell pellet was resuspended in 100 μl of complete medium containing RPMI 1640, penicillin, and streptomycin, 10% heat-inactivated human AB^+ serum (Sigma Chemical Co., St. Louis, MO), 10 U/ml rIL-2 (Ajinomoto, Kawaski, Japan), and 5 U/ml rIL-4 (Genzyme, Cambridge, MA). The cell pellet was placed in culture with phytohemagluttinin-P (PHA-P) (Wellcome, Altringham, U.K.; 1 μg/ml) in a 96-well V-bottom cell culture cluster (Costar, Cambridge, MA) in 37°C humidifed atmosphere containing 6% carbon dioxide. The cells were fed at day 3 with complete medium and at day 7 with PHA-P and irradiated (5000 rad) allogeneic PBMC (1×10^5/well). Stimulation with PHA-P and irradiated feeders was repeated every 10 days until a T cell line was considered established, usually after 3 to 4 wk of cultivation. These conditions are similar to those used previously by us to establish IEL T cell lines (7). Two peripheral blood T cell lines were established by similar means from PBMCs of different normal donors.

Antibodies

The following mAbs were used for the primary T cell labeling: phycoerythrin (PE)-conjugated anti-CD4 or -CD8 (Becton Dickinson, San Jose, CA), FITC-conjugated anti-TCR-$\alpha\beta$ (Becton Dickinson) or anti-CD3 (Dako, Carpinteria, CA), and biotin-conjugated anti-CD8 (Coulter, Hialeah, FL) or CD45RO (Dako). Phycoerythrin- and FITC-conjugated mouse IgG1 and IgG2 Abs were used as negative controls (Exalpha, Boston, MA). The OKT3 mAb (IgG2), as a purified culture supernatant, was used in redirected cytolysis assays. Immunostaining was performed using the following Abs: anti-CD3 (Dako), peroxidase-conjugated rabbit anti-mouse Ig antiserum (Dako), CD45RO (Zymed, San Francisco, CA), and CD43 (Becton Dickinson).

Cell lines

Intestinal epithelial cell lines (Caco-2 and HT29), a hepatocyte cell line (HepG2), two fibroblastic cell lines (human foreskin fibroblasts and KD), and P815 mouse mastocytoma cell line were maintained in either RPMI 1640 or DMEM (Mediatech) supplemented with 10% FBS (Sigma Chemical Co.), L-glutamine, nonessential amino acids (BioWhittaker), and penicillin and streptomycin (100 U/ml; BioWhittaker). The cells were harvested from the culture flask using Enzyme Free cell dissociation solution (Specialty Media, Lavallette, NJ) to preserve the cell surface proteins recognized by the effector cells during the functional assays.

Flow cytometric analysis

Three-color immunofluorescence staining was performed as previously described (20). Briefly, 1 to 2×10^6 T cells from cell lines were washed twice with ice-cold buffer (PBS with 0.02% (w/v) azide and 1% BSA (Fraction V; Sigma Chemical Co.)). The cells were then incubated with 1 μg of the directly conjugated mAbs for 30 min on ice. After two washes, the cells were incubated with avidin-Cy5 (Biological Detection Systems, Pittsburgh, PA) for 30 min on ice and fixed with 1% (w/v) paraformaldehyde in PBS/0.02% azide. The samples were analyzed on a Coulter EPICS Elite flow cytometer (Coulter).

Histology and immunohistochemistry

One of the subjects, PSC-1, had undergone liver and common bile duct biopsies for evaluation of the primary sclerosing cholangitis. These biopsies were studied to assess the histopathologic presence of T cells in these sites. The specimens were fixed in 10% buffered formalin, routinely processed, and embedded in paraffin. Serial 4-μm thick tissue sections were prepared. Several sections of each slide were stained with hematoxylin and eosin and evaluated by a gastrointestinal surgical pathologist (J. R. Turner). For immunohistochemistry, tissue sections were deparaffinized, incubated in methanolic peroxide (3% H_2O_2: methanol 1:5, v/v) for 30 min, and washed. For CD3 immunostaining, tissue sections were incubated for 15 min at 37°C in 50 mg/dl trypsin (Sigma Chemical Co., catalog no. T-8128) in 50 mM Tris-HCl, pH 7.8, with 9 mM $CaCl_2$ to expose Ag sites. These were then placed in 50 mM Tris-HCl, pH 7.6, with 3% porcine serum and incubated sequentially with anti-CD3 (1:200 dilution), and peroxidase conjugated rabbit anti-mouse Ig antiserum (1:40 dilution). Peroxidase activity was detected using the $LSAB^+$ peroxidase detection kit (1:2 dilution). For CD45RO immunostaining, tissue sections were first incubated for 5 min at 37°C in target unmasking fluid (Signet, Dedham, MA) to expose Ag sites. No pretreatment was necessary for CD43 immunostaining. Staining for CD45RO (1:50 dilution) or CD43 (1:200 dilution of Leu-22) was performed using the Ventana ES slide staining system (Ventana Medical Systems, Tucson, AZ) according to the manufacturer's protocol. All immunostained slides were counterstained with hematoxylin, dehydrated, and mounted with Permount (Fisher Scientific, Springfield, NJ).

Redirected lysis and cytotoxicity assays

Redirected lysis assays were performed using the P815 mouse mastocytoma cell line as target cells. One × 10^6 target cells were labeled with 1 mCi of [^{51}Cr]$NaCr_2O_3$ (New England Nuclear, Boston, MA) for 1 h at 37°C. After washing twice in RPMI, the targets were plated at 10^4 cells/well in a 96-well U-bottom cell culture cluster (Costar) with effector T cells that had been previously incubated with purified OKT3 culture supernatant for 20 min at room temperature. Varying combinations of E:T ratios ranging from 2.5:1 to 200:1 were incubated under these conditions. Maximal and minimal release labeling of [^{51}Cr] from target cells was assessed by suspension in either Triton X-100 (1% v/v) or RPMI alone, respectively. All experiments were performed in triplicate in a reaction volume of 200 μl. The assay plate was incubated for 4 h at 37°C after which 100 μl of supernatant was collected and assayed in a gamma counter. Specific killing of the Caco-2 cell line, an intestinal epithelial cell line, was assessed by labeling 1×10^6 target cells with ^{51}Cr as described above and using 10^4 Caco-2 cells/well in a 96-well U-bottom plate as targets in quadruplicate for the normal CBD-derived cell line as effectors and triplicate for the PSC-1 CBD-derived line as effectors at E:T ratios ranging from 2.5:1 to 200:1. The percent lysis was calculated using the formula:

$$\% \text{ lysis} = \frac{(\text{samples release cpm} - \text{spontaneous release cpm})}{(\text{maximum release cpm} - \text{spontaneous release cpm})} \times 100.$$

The cytotoxic activity was expressed in LU (21) in the linear range of lysis between 15 and 85% using the formula:

$$\text{LU} = \frac{\text{In (1\% lysis)}}{\text{In (E:T)}}.$$

LU, expressed as mean ± SD for approximately 30% lysis in the midlytic range, were compared using Student's *t* test for unpaired samples.

Proliferation assays

Assays were performed in 96-well flat-bottom plates. Epithelial and fibroblast cell lines were grown to confluency in 75-cm^2 flasks. They were dissociated using nonenzymatic cell dissociation solution, irradiated with 7500 rad, and resuspended in 100 μl complete RPMI containing 10% human serum but without cytokines. Each well of the assay plate received 1/100 of the cells contained in the original 75-cm^2 flask. T lymphocytes

were seeded at 10^5 cells per well in the same medium. Control wells received no T cells. After 48 h incubation at 37°C, wells were pulsed with 0.5 μCi [^{3}H]thymidine. After another incubation of 18 h, cells were harvested on filter mats (Walac, Turku, Finland) and read in a scintillation counter.

Complementarity-determining region 3 (CDR3) analysis of TCRB chains

Three to five × 10^6 T cells from each of the three bIEL and a peripheral blood T (PBT) control cell lines were snap frozen for preparation of cDNA and subsequent PCR amplification as previously described (7–9). Briefly, RNA was extracted from the frozen cell pellets using RNAzol-B (Cinna Scientific, Friendswood, TX) according to the manufacturer's instructions. cDNA was synthesized by incubating the RNA with 200 U Moloney murine leukemia virus (MMLV) reverse transcriptase (Life Technologies, Grand Island, NY) in the presence of 45 U RNAsin (Pharmacia), 10 μM oligo(dT) (Perkin-Elmer, Foster City, CA), 0.5 mM deoxyribosylnucleotide triphosphates (dNTPs), and buffers provided by the manufacturer (Life Technologies) in a reaction volume of 20 μl for 30 min at 37°C and then for 15 min at 42°C. The integrity of cDNA was assessed by PCR amplification with primers specific for β_2-microglobulin as previously described (7–9). cDNA was PCR-amplified in a series of 10-μl reactions that contained a consensus TCR β-chain-constant region (TCRBC) antisense primer (5 pmol) (8, 9), a TCRBV 1–24 region family-specific primer (5 pmol) (8, 9), 0.1 mg/ml BSA, 0.01 mM dNTPs, 1.5 mM $MgCl_2$, 0.25 U Taq polymerase (Promega Corp., Madison, WI), and 1 μCi of [α-^{32}P]dCTP (sp. act., 3 Ci/μmol) in thermophilic buffer provided by the manufacturer (Promega). Each reaction consisted of 32 cycles, in a MJ Research Thermal Cycler (Cambridge, MA) as follows: 94°C for 20 s, 55°C for 30 s, and 72°C for 60 s. The last cycle was followed by a final extension period of 10 min at 72°C, then chilled to 4°C. The labeled PCR products were heat denatured for 10 min at 75°C and loaded onto a 6% polyacrylamide gel, prewarmed to 50°C, cast in NUNGENEration Sequencing System (Owl Scientific, Cambridge, MA) as previously described (8, 9).

Subcloning and sequencing of cDNA from PCR-amplification products

cDNA was PCR-amplified in a 20-μl reaction mixture that contained 10 pmol of the TCRBC antisense primer, 10 pmol of the appropriate TCRBV primer, 0.1 mg/ml BSA, 0.2 mM dNTPs without added radiolabeled dCTP, 1.5 mM $MgCl_2$, and 0.5 U Taq polymerase in thermophilic buffer. Each reaction consisted of 32 cycles as described above. A second round of PCR amplification was performed in a 50-μl reaction volume containing 2 μl of the primarily amplified cDNA, 25 pmol of a consensus nested antisense TCRBC primer (8, 9), and an additional 25 pmol of the relevant TCRBV primer in a reaction mixture containing 0.1 mg/ml BSA, 0.2 mM dNTPs, 1.5 mM $MgCl_2$, and 1 U Taq polymerase in thermophilic buffer for 25 cycles, using amplification conditions described above except for a final extension of 30 min. The PCR products were purified using Wizard PCR DNA Purification System (Promega) and 10 ng of cDNA cloned using the TA cloning Vector (Invitrogen Corp., San Diego, CA) according to the manufacturers' protocols. Subsequently, *Escherichia coli* strain InvαF' were transformed by heat shock and miniprepped with Wizard Miniprep DNA Purification System (Promega) according to the manufacturer's protocol. Plasmids from randomly isolated bacterial colonies were subjected to dideoxy-DNA sequencing using the nested antisense Cβ primer (2 pmol), 5 μCi of [γ-^{35}S]dATP (sp. act., 1.2 Ci/μmol), and 4 U of Sequenase (United States Biochemical, Cleveland, OH) per set of four sequencing reactions. The samples were heat denatured at 75°C for 10 min and loaded onto a prewarmed 6% polyacrylamide DNA sequencing gel.

Elution, subcloning, and sequencing of amplified cDNA from CDR3 display gels

Bands of interest were excised from the CDR3 display gel, each with a fresh razor blade. The DNA was eluted by soaking overnight in 0.5 M ammonium acetate, 0.1 mM EDTA, and 0.1% SDS at 37°C. After ethanol precipitation, the DNA was reamplified by PCR for 25 cycles, as described above, using the nested TCRBC primer. PCR products were then subcloned into the TA cloning vector (Invitrogen) and colonies picked at random, miniprepped, and sequenced.

Colony hybridization with clonotypic primers

After PCR amplification of cDNA from line PSC-2 using TCRBC and TCRBV16 primer pairs, PCR products were cloned using the TA vector, as described above. Colonies were lifted onto supported nitrocellulose membrane (Schleicher & Schuell, Keene, NH). An antisense primer specific for the NDN region of the primary nucleotide sequence of the TCRBV16-QETFY-TCRBJ2S1 chain (TGTAAAATGTTTCTTGGC) was end labeled with [γ-^{32}P]dATP using 20 U polynucleotide kinase (New England Biolabs, Beverly, MA) according to the manufacturer's protocol. Twenty × 10^6 counts/ml of the NDN-specific probe were added to 50 ml of prehybridization solution (5× Denhardt's solution, 5× SSC, 0.1% SDS), and hybridization was performed at 37°C overnight. The membrane was washed twice in 5× SSC/0.1% SDS and then once in 2× SSC/0.1% SDS. All washes were for 20 min at room temperature. The autoradiogram was developed after 24 h of exposure. Plasmid DNA was prepared from hybridizing colonies and sequenced as above.

Interpretation of sequences

Using the Clone software program, primary nucleotide sequences were translated into amino acid sequence. The TCRBV family and TCRBJ sequences were defined according to published nomenclature (22–25). The NDN regions were defined as the sequence between the last identifiable TCRBV amino acid to the first identifiable amino acid belonging to a recognized TCRBJ family. TCR gene segments were named according to World Health Organization nomenclature (26).

Results

Establishment and phenotypic characterization of CBD-derived cell lines

Cells derived from brush samples obtained during ERCP of three subjects were cultivated initially in medium containing rIL-2, rIL-4, and PHA-P without added allogeneic feeder cells to avoid preferential expansion of T cells on the basis of allogeneic recognition. After 1 wk in culture and every 10 days thereafter, the cells were restimulated with rIL-2, rIL-4, PHA-P, and irradiated allogeneic PBMCs. After 3 to 4 wk of stimulation, adequate numbers of cells were obtained for the isolation of RNA and, in the case of three samples (PSC-1 and -2 and normal), for phenotypic and functional studies. For unknown reasons, the T cell line derived from the PSC/UC sample experienced a crisis during the fourth week of cultivation and could not be retrieved. As a result, only the PSC-1, PSC-2, and normal sample were available for phenotypic analysis (Table I). In these samples, all the cells were CD3$^+$, TCR-αβ$^+$, and CD45RO$^+$. Nearly all of the lymphocytes were either CD4$^+$ or CD8$^+$ with CD4:CD8 ratios ranging from 0.1 to 0.5 for the PSC-1 and PSC-2 samples, respectively, and 1.2 for the normal sample-derived line. Neither double-positive (CD4$^+$CD8$^+$) nor double-negative (CD4$^-$CD8$^-$) cells could be detected in any sample. Thus, T cells that were predominately TCR-αβ$^+$ and either CD4$^+$ or CD8$^+$ could be obtained and expanded from both normal and abnormal bile duct samples.

Immunohistologic characterization of CBD-associated lymphocytes

In case PSC-1, biopsies of both the liver and CBD were obtained. The findings in the liver were not diagnostic of PSC, but included features of PSC such as portal edema and fibrosis with lymphocytic infiltration, focal bile duct proliferation, cholangitis and pericholangitis, reduced numbers of interlobular bile ducts, and damage to the limiting plate. Well-developed lamellar fibrosing lesions were not present. The CBD biopsy was notable for lamina propria fibrosis, a marked lamina propria lymphocytic infiltrate, and numerous IELs (Fig. 1, *A*, *B*, and *D*). Immunostaining for lymphocytes in the CBD biopsies showed that many lymphocytes of the lamina propria and IELs were positive for the T cell marker CD3 (Fig. 1, *C* and *E*, respectively). An identical staining pattern was observed with anti-CD43 and anti-CD45RO mAbs (data not shown). These studies suggest that the cultivated CBD-derived T cells originated from the CBD epithelium and/or lamina propria.

CDR3 analysis of CBD-derived cell lines

A major characteristic of freshly isolated human intestinal IELs and T cell lines obtained from normal human intestinal IELs is that

Table I. *Flow cytometric analysis of the phenotype of CBD-derived T-cell lines*[a]

Marker	Subject		
	PSC-1	PSC-2	Normal
CD3	98.9%	99.7%	99.3%
TCR-$\alpha\beta$	99%	99%	97.3%
CD45RO	91.8%	99%	98.3%
CD8	70.3%	90%	44.8%
CD4	32.1%	9.2%	54.5%
CD8:CD4	2.2	9.8	0.8

[a] Flow cytometric analysis was performed on three of the CBD-derived T cell lines. One to two million cells were used for each Ab-staining reaction.

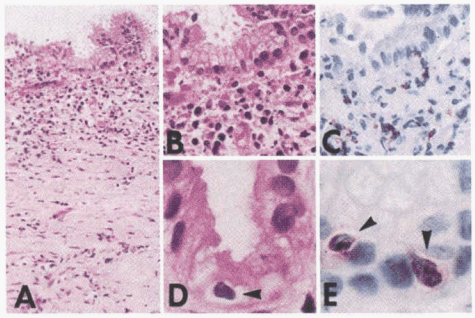

FIGURE 1. Histopathology of a common bile duct stricture in a patient with PSC not associated with UC (PSC-1). Extensive submucosal fibrosis is present within the wall of the common bile duct at the stricture site (*A*). At intermediate magnification, numerous IEL and lamina propria lymphocytes can be seen (*B*), many of which stain positively for the T cell marker CD3 (*C*). High power examination of biliary epithelium shows abundant IELs (*D*, arrow), which also stain positively for CD3 (*E*, arrows). Staining with anti-CD45RO and anti-CD43 mAbs was identical to the anti-CD3 staining (data not shown).

they are derived from the expansion of a limited number of T cell clones (7–10). To assess whether oligoclonality is also a characteristic of CBD-derived T cell lines, the complexity of TCR-$\alpha\beta$ usage of the T cell lines was initially determined by analysis of the distribution of CDR3 lengths. cDNA obtained from three CBD-derived T cell lines (PSC-1, PSC/UC, and normal) was PCR-amplified with a family of primers specific for each of 24 different TCRBV families in conjunction with a consensus Cβ primer in the presence of [α-^{32}P]dCTP. The PCR-amplified products were resolved by denaturing polyacrylamide electrophoresis. As can be seen in Figure 2*A*, the TCRB amplification products derived from all three CBD-derived cell lines exhibited a different pattern of TCRB chain usage, which was markedly skewed. Only a few TCRBV families were represented without any preferential TCRBV usage and, within these families, a few scattered bands were observed. This pattern was in marked contrast to the CDR3 length analysis of a T cell line obtained in a similar manner from normal peripheral blood (Fig. 2*B*). In the latter case, a cluster of 6 to 10 bands was observed whose intensity followed a normal distribution (27). Thus, the CDR3 analysis of TCRB chains of CBD-derived cell lines was consistent with an oligoclonal population.

TCRB sequence analysis of CBD-derived T cell lines

To confirm whether the CBD derived T cell lines originated from a limited number of T cell clones, the total TCRBV family PCR amplification products were cloned and randomly sequenced. PCR amplification products derived from TCRBV families that were identified as dominant by CDR3 analysis were subcloned into the TA cloning vector, and plasmid DNA from randomly isolated bacterial colonies was sequenced. Table II shows the sequencing results for several TCRBV families analyzed in this manner. In all three CBD-derived cell lines (PSC-1, PSC/UC, and normal), each of the TCRBV amplification products analyzed comprised a limited number of TCRBV sequences. Indeed, for several of the TCRBV families, only a single TCRB clone was identified. Although no preferential TCRBV family was utilized and no characteristic junctional motif was identifiable, the sequencing analysis indicated that T cells derived from the common bile ducts of normal donors and subjects with PSC, either alone or complicating UC, like normal human intestinal IELs, were pauciclonal.

Detection of persistent TCRB sequences among CBD-derived T cell lines

In one case, a second longitudinal sample was available from the same individual approximately 1 yr after the first sample. The phenotype of this T cell line (PSC-2) is shown in Table I. To determine whether T cell clones that were present in sample PSC-2 were also present in sample PSC-1, two strategies were used. First, CDR3 analysis was performed simultaneously on cDNA from the PSC-1 and PSC-2 samples. Bands from PCR products that coresolved on a DNA sequencing gel, indicating the presence of clones of similar length, were extracted; the DNA was eluted, reamplified, and subcloned into the TA cloning vector; and random sequencing was performed. Table III shows the results of several bands examined in this manner. In both samples, DNA sequencing revealed that all bands analyzed were derived from the expansion of one or several TCRB clones, confirming the data in Table II on the total TCRBV PCR products. In the case of coresolving dominant bands within the TCRBV14 amplification product, identical TCRB sequences could be identified. In sample PSC-1 and sample PSC-2, 7 of 7 TCRB and 4 of 6 TCRB sequences, respectively, consisted of identical nucleotide sequences, which translated as TCRBV14-FTGTGH-TCRBJ1S2. This indicates that a dominant TCRBV14 T cell clone expressing this TCRB chain was persistent within the CBD over the period of approximately one year. A second series of experiments was designed to look specifically for evidence of persistence of the T cell clone bearing the TCRB chain TCRBV16-QETFY-TCRBJ2S1 (see Table II), which was expanded in PSC-1. The total TCRBV16 PCR amplification products from sample PSC-2 were cloned into the TA cloning vector and, after transformation, those colonies that hybridized with a primer specific for the NDN region of the dominant TCRBV16 clone in PSC-1 were sequenced. As seen in Figure 3, the TCRBV16-QETFY-TCRBJ2S1 sequence could be identified among the TCRBV16 amplification products of sample PSC-2. Thus, TCRB clones can be identifed in CBD-derived T cells that persist over a period of at least 1 yr.

Functional analysis of CBD-derived cell lines

The phenotypic studies of the CBD-derived cell lines from both the normal and PSC subjects suggested that they shared several features in common with intestinal IELs. To determine whether the TCR/CD3 complex of the CBD-derived cell line was functional and whether it shared with intestinal IELs cytolytic activity against intestinal epithelial cells, the cytolytic function of the CBD-derived T cells from PSC-1 and the normal subject was examined. For comparison, this cytolytic activity was compared with a PBT cell line from an unrelated donor. Both of the CBD-derived cell lines exhibited significant lysis of the P815 mastocytoma cell line in a redirected lysis assay using cross-linking with an anti-CD3 mAb (Table IV). As expected, the cytolytic activity was significantly greater after cross-linking, compared with the activity in the absence of anti-CD3 cross-linking. Both of the CBD-derived lines

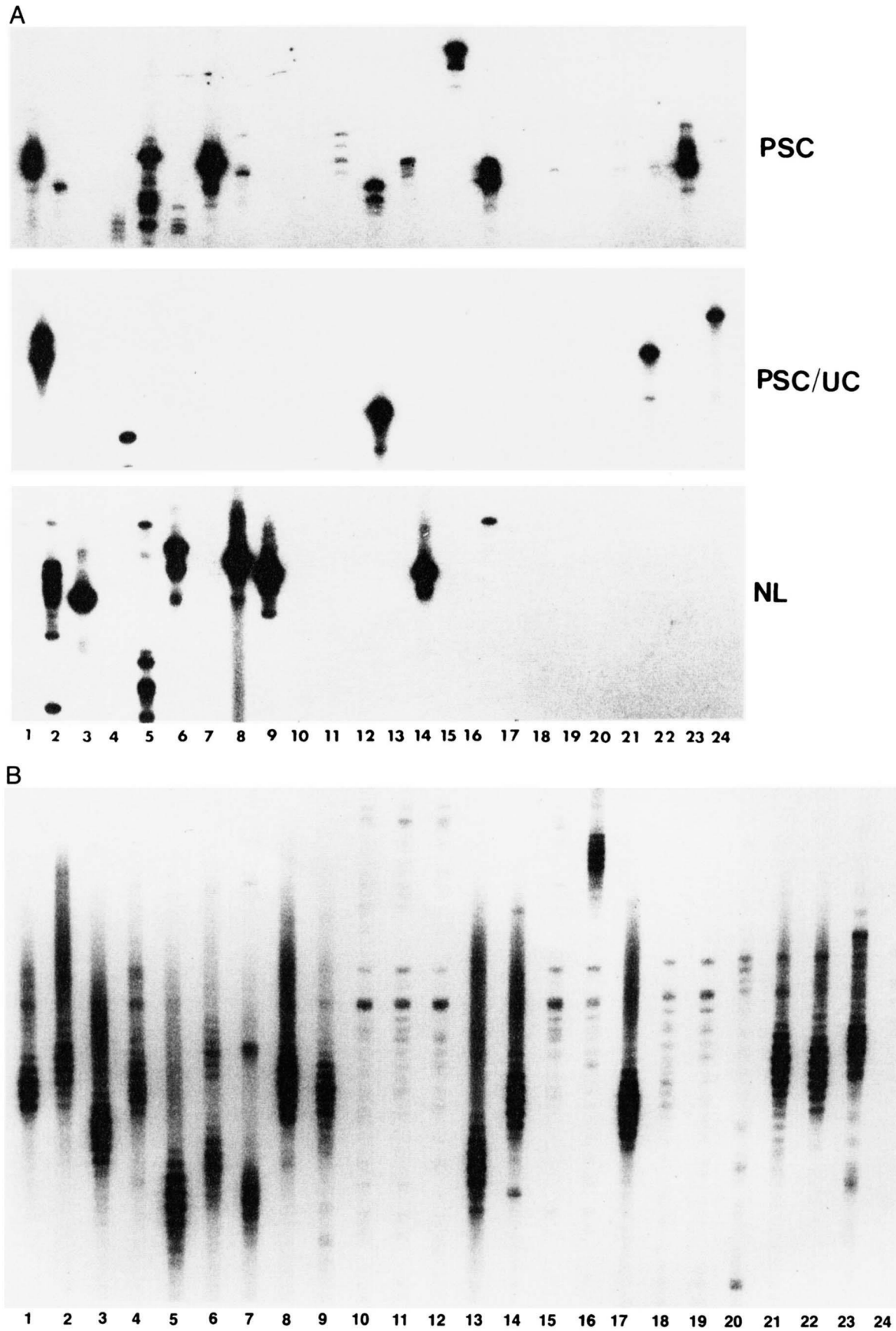

FIGURE 2. CDR-3 length analysis of cDNA from three of the CBD-derived T cell lines. CDR3 length displays were performed as described in *Materials and Methods* on cDNA prepared from three of the CBD-derived T cell lines (*A*) and a normal peripheral blood T cell line (*B*) after 3 to 4 wk in culture. The TCRBV primer used for each reaction is shown.

Table II. *TCRB sequences obtained from CBD-derived T cell lines*[a]

Subject	TCRBV	NDN Region	TCRBJ	Frequency[b]
PSC-1	5	LEPI	1S2	2/5
	5	LEGL	2S3	1/5
	5	NPLGE	2S5	1/5
	5	LWGRESG	2S7	1/5
	16	QETFY	2S1	5/6
	16	QETFC	2S1	1/6
PSC/UC	12	RATGGI	1S1	4/6
	12	ESGSV	2S5	2/6
	21	TR	S2S	18/18
Normal	3	RPTGV	2S2	10/10
	14	LLAGAA	2S3	4/6
	14	VRGLG	1S3	2/6

[a] PCR products from TCRBV-specific amplifications were cloned and randomly sequenced using cDNA from three CBD lines. Amino acid sequences in single-letter code are shown for the NDN region. The TCRBV and truncated TCRBJ regions are described numerically according to References 22 through 25.
[b] Indicates proportion of total sequences identified.

also exhibited significant lysis of the Caco-2 cell line, an intestinal epithelial cell line (Table V). This Caco-2-directed lysis was greater than the lysis of Caco-2 observed with the PBT line (Table V). However, considering the lower overall cytolytic potential of the PBT cell line as defined by the redirected lysis assay (Table IV), it can be concluded that both the CBD-derived and PBT cell lines contained cytolytic effector cells capable of recognizing intestinal epithelial cells.

To determine whether the recognition of intestinal epithelial cells observed in the cytolysis assays was preferentially directed at intestinal epithelial cells, the normal CBD-derived cell line and PBT cell line were examined in a proliferation assay with a panel of cell lines that included two intestinal epithelial cell lines (Caco-2 and HT29), a hepatocyte cell line (HepG2), and two fibroblastic cell lines (human foreskin fibroblasts, and the lip fibroblast cell line, KD). As can be seen in Figure 4, both the CBD-derived cell line and PBT cell line exhibited strong proliferation in response to Caco-2 and HT29, but not to HepG2, human foreskin fibroblasts, and KD. These studies suggest that the CBD-derived and PBT cell lines contain effector cells that are directed preferentially against T cells of intestinal epithelial cell origin.

Discussion

We have shown that T cells can be isolated and expanded in vitro from normal and abnormal CBDs and that they share several important features with intraepithelial T cells from other sites (1–3,

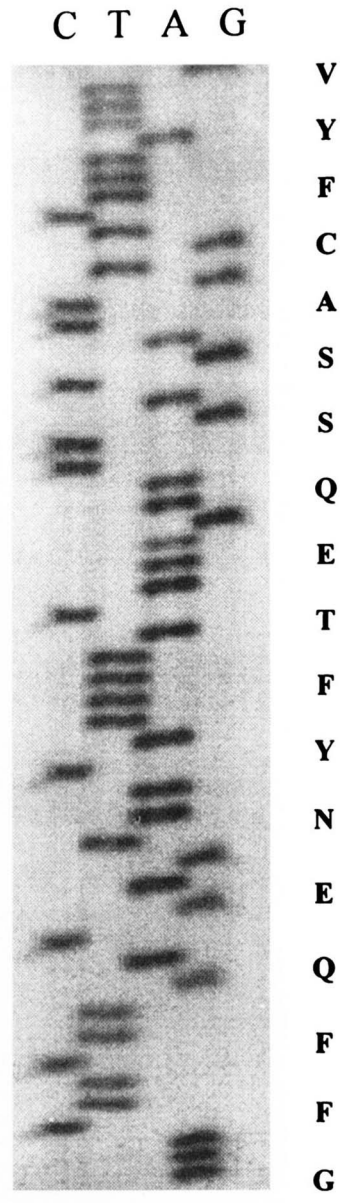

FIGURE 3. Sequencing of persistent TCRBV16 clone in sample PSC-2. Sequencing gel of a TCRB chain sequence from the TCRBV16 amplification product from sample PSC-2, which was also present 10 mo earlier in sample PSC-1. The sequenced nucleotide is shown for each lane at the *top*, and the translated amino acid sequence in single-letter amino acid code is shown on the *right*.

Table III. *TCRB sequences obtained from DNA eluted from dominant bands of CDR3 displays of the lymphocyte lines PSC-1 and PSC-2*[a]

PSC-1 (November 1994)				PSC-2 (September 1995)			
TCRBV	NDN region	TCRBJ	Frequency[b]	TCRBV	NDN region	TCRBJ	Frequency[b]
3	LRQTL	2S7	5/7	3	WDR	1S1	4/4
3	LSGGD	2S1	2/7				
8	TQDLL	2S3	5/5	8	LAGEW	2S1	4/6
				8	RLDRV	1S4	2/6
14	**FTGTGH**	**1S2**	7/7	**14**	**FTGTGH**	**1S2**	**4/6**
				14	LTGG	2S7	2/6
23	LDTS	2S1	4/4	23	LGTGD	2S2	6/6

[a] cDNA, prepared from CBD-derived T cell lines PSC-1 and PSC-2 at time points separated by 10 mo (November 1994 and September 1995, respectively) were subjected to CDR3 length analysis as described in *Materials and Methods*. Bands of the same length were identified and the DNA eluted. After reamplification, the cDNA was cloned and random bacterial isolates sequenced. Results are shown for bands displayed within the TCRBV3 -8, -14, and -23 amplification products. Amino acid sequences in single-letter code are shown for the NDN region. Shared reiterative clones are indicated by bold print.
[b] Indicates proportion of total sequences identified.

Table IV. *Cytotoxic activity by CBD-derived cell lines and PBT line by redirected lysis*[a]

	CBD-Derived Line (LU)	Control PBT Line (LU)	*p*
Normal CBD line	90 ± 70	45 ± 44	<0.05
	(23.5 ± 3.6)	(6.2 ± 24)	
PSC-1 line	102 ± 16	30 ± 20	<0.01
	(16 ± 33)	(4.0 ± 7)	

[a] Redirected lysis of ^{51}Cr-labeled P815 mastocytoma cell line expressed as LU (mean ± SD) × 10^3 in the midlytic range by CBD-derived cell lines from the normal donor and PSC-1 donor in comparison with a normal PBT cell line using an anti-CD3 Ab for cross-linking. Data given in parentheses indicate LU without added anti-CD3 Ab. The *p* value is shown for the comparison between the CBD-derived lines and the PBT line.

Table V. *Cytotoxic activity by CBD-derived cell lines and PBT line against Caco-2 cell line*[a]

	CBD-Derived Line (LU)	Control PBT Line (LU)	*p*
Normal CBD line	129 ± 8	51 ± 9	<0.05
PSC-1 line	113 ± 30	34 ± 1	<0.01

[a] Cytotoxicity of ^{51}Cr-labeled Caco-2 cells expressed as LU (mean ± SD) by the CBD-derived cells line from the normal donor and PSC-1 donor in comparison with a normal PBT cell line as described in Table IV. The *p* value is shown for the comparison between the cytotoxicity of the CBD-derived cell lines and the PBT line.

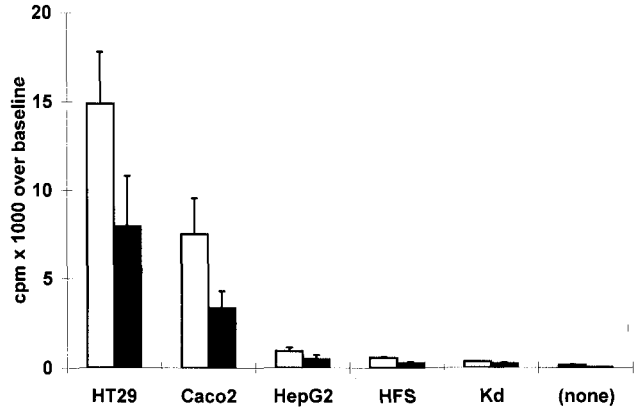

FIGURE 4. Proliferation of CBD-derived and PBT cell lines. CBD-derived T cell line from a normal CBD and a PBT cell line were cocultivated with irradiated stimulator cell lines in 96-well flat-bottom plates for 48 h in quadruplicate and [^{3}H]thymidine uptake assessed after 18 h as described in *Materials and Methods*. The *y*-axis shows the cpm × 100 over baseline representing [^{3}H]thymidine uptake of the irradiated stimulator cells without added T cells. "None" represents the results of T cell proliferation without added stimulator cells. The means ± SD are indicated for each stimulator cell line. Open bars represent the CBD-derived cell line and filled bars the PBT cell line. Both the CBD-derived and PBT cell lines exhibited significantly greater proliferation to Caco-2 and HT29 than to HepG2, human foreskin fibroblast, and KD at a level of $p < 0.01$.

7–10). Phenotypically, the CBD-derived cell lines that we established were predominantly TCR-$\alpha\beta^+$ and either CD4$^+$ or CD8$^+$, with a slight predominance of CD8$^+$ T cells, especially in the PSC samples. These phenotypic characteristics are similar to large intestinal IELs (6). Although T lymphocytes have been observed for decades in the wall of the CBD, the TCR usage, accessory phenotype, and function of these cells have not been addressed. It is known that in the liver and gallbladder of subjects with PSC and primary biliary cirrhosis that CD4$^+$ and CD8$^+$ T cells are associated with the bile duct lesions observed in these diseases (14, 15, 17). Furthermore, as shown by the immunostaining, T cells can insinuate between biliary epithelial cells, in a manner similar to IELs in the intestine, lending potential clinicopathologic relevance to our observations of a slight CD8 predominance in the two PSC samples.

TCRB analysis showed that the T cell lines described here were composed of a limited number of T cell clones. The pauciclonality was observed in both the cell lines derived from the normal donor and from donors PSC and PSC complicating UC. This indicates that pauciclonality is likely a common feature of bIELs, regardless of disease activity, and parallels the oligoclonality of T cells in the liver of subjects with primary biliary cirrhosis as reported by Diu et al. (28). Although no common TCRBV region family was utilized by the cells in our study, it is possible that in a larger sample, disease-related TCR motifs may be definable. Oligoclonality (or pauciclonality) is a common feature of epithelium-associated TCR-$\alpha\beta^+$ T cells of the skin (29, 30) and gut of humans (7–10) and mouse (31). The studies described here suggest that the lymphocytes associated with the human CBD also likely share this attribute.

The Ags recognized by IELs, which may provide important insights into the origin of the oligoclonality, are presently unknown. These Ags may represent either a limited number of epithelial Ags or dominant luminal Ags within the bile duct. In mouse intestine, since TCR-$\alpha\beta^+$ IELs are significantly diminished in the absence of luminal bacteria (32) and/or β_2-microglobulin (33), it might be argued that the target Ag is derived from a luminal Ag in the context of an MHC class I-like molecule. Furthermore, others have previously suggested that the epithelial colonization by a limited number of T cell clones is a stochastic event, since littermates of congenic mouse strains exhibit different intestinal IEL T cell clones (31). In this regard, we have found, through longitudinal analysis, that certain TCR-β clones are persistent over at least a 1-yr period within the bile duct samples analyzed here. IEL persistence has also been previously observed for human TCR-$\gamma\delta^+$ intestinal IELs (11, 34). These data argue against a completely stochastic model for bIEL localization and suggest that specific CBD-associated Ags may be present that are responsible for at least some of the persistent clonal expansions. Since the persistent clonal expansions were observed in the diseased sample, it may be speculated that these are disease-related Ags. Longitudinal studies of normal CBD samples are, however, not generally accessible to address whether this is unique to disease states.

A final characteristic of the CBD-derived cell lines examined here was the apparent preferential recognition of epithelial cell lines. The tissue-specific function and cognate Ag of IELs are unknown. Human intestinal IELs, which are predominantly CD8$^+$, will bind to and lyse tumor cell lines of epithelial cell origin (13). In addition, it has been shown that CD8$^+$ PBT cells will specifically proliferate to intestinal epithelial cells (35). Taken together, these observations suggest that both peripheral blood and intestinal epithelium contain CD8$^+$ T cells that recognize and respond to intestinal epithelial cells. The studies described here show that CBD-derived T cells also share this characteristic in that they exhibit lysis of and selective proliferation to intestinal epithelial cell lines.

In summary, we have shown that T cells can be obtained from normal and diseased human CBDs, which exhibit several features in common with intestinal IELs. This approach to the analysis of CBD T cells will provide a unique opportunity for investigation of autoimmune and infectious diseases that target this previously inaccessible epithelial site.

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