GUT IMMUNOLOGY

# Heterogeneity of intraepithelial lymphocytes in refractory sprue: potential implications of CD30 expression

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*Gut* 2002;**51**:372–378

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Accepted for publication 18 December 2001

**Background:** Refractory sprue is defined as primary or secondary failure to respond to a gluten free diet in patients with coeliac disease-like enteropathy and may signify cryptic or overt enteropathy associated T cell lymphoma.
**Aims:** To study in detail jejunal morphology and immunophenotypes in patients with refractory sprue in the search for features that might be useful to predict prognosis.
**Patients:** Seven patients are described, representing all such cases identified in our hospital over a 13 year period.
**Methods:** Biopsy and/or surgical resection specimens were examined by morphology, immunohistochemistry, including enzymatic and immunofluorescent detection, and molecular biology.
**Results:** All patients had phenotypically abnormal intraepithelial lymphocytes (IELs) that lacked CD8, T cell receptor αβ (or γδ), and/or expressed CD30 in addition to variable expression of the natural killer cell receptor CD94. A monoclonal T cell population was present in six cases, data from the seventh being inconclusive. Three patients had overt lymphoma with CD30+ tumour tissue intervening between intact mucosa that contained neoplastic IELs. Intriguingly, CD30+ IELs were observed both a long way away from, and in direct continuity with, the tumours in these patients. Such CD30+ cells were hardly detected in patients without tumours, two of which are in good health several years after the initial diagnosis.
**Conclusions:** Our data suggest that abnormal IELs in patients with refractory sprue are phenotypically heterogeneous. CD30 expression by these cells may indicate a worse prognosis, including the occurrence of overt lymphoma.

Refractory coeliac disease is defined as primary or secondary failure to respond to a gluten free diet (GFD) in patients whose clinical, histological, and serological features suggest coeliac disease.[1] Refractory sprue is often used to characterise such patients when formal proof of a coeliac background is lacking, such as failure to produce antibodies to tissue transglutaminase.[1–3] Elevated endomysial antibody (EMA) titres have proved especially important to exclude other causes of enteropathy whereas the antigliadin antibody (AGA) titre may also be elevated in other conditions.[1]

Patients with refractory coeliac disease may have phenotypically normal intraepithelial lymphocytes (IELs) and benefit from immunosuppressive treatment.[2] However, many patients with refractory sprue have phenotypically abnormal IELs defined by loss of T cell antigens such as surface CD3ε (cytoplasmic CD3ε being present), CD8, or the T cell receptor (TCR), and in addition appear to be monoclonal.[3] Such IELs are considered neoplastic and have been suggested to represent a cryptic variant of enteropathy associated T cell lymphoma (EATL).[2] Refractory sprue usually has a poor prognosis, mainly due to uncontrolled malabsorption.[1 3] However, the condition may also be the initial manifestation of overt EATL that has an even poorer prognosis.[4] Jejunal ulcers often develop in the course of refractory sprue, and they may represent the initial stage of overt EATL.[5–7] The tumour cells of EATL virtually always express CD30 but this marker has not been described on the phenotypically abnormal IELs adjacent to CD30+ tumours[8] or in refractory sprue. Firm evidence that EATL originates from IELs, with or without intermediate ulcer formation, has not been obtained, although most data indicate that this is the case.[2 8–10]

What drives the lymphoma development in coeliac disease remains unknown. One report suggested that individuals heterozygous for DRB1*0304 are particularly prone to develop EATL.[11] Notably, Murray and colleagues[8] speculated that patients with continued exposure to gluten and only mild symptoms escaping suspicion of coeliac disease represent an EATL risk group. We have followed patients with refractory sprue since 1988 and have identified seven cases with abnormal IELs, four of which had developed overt lymphoma. The clinical, morphological, and immunphenotypic heterogeneity of this patient cohort is described. The data obtained suggest that CD30 expression by IELs of patients with refractory sprue indicates a poor prognosis, including the presence of overt EATL.

## MATERIAL AND METHODS

### Patient histories

Clinical characteristics of the seven patients are summarised in table 1.

#### Patient No 1

Patient No 1 was healthy until the age of 54 years when he was admitted to hospital because of persistent diarrhoea and a 20 kg weight loss. Coeliac disease was diagnosed on the basis of a flat mucosal lesion and increased AGA titres. He did not respond to a GFD alone and was additionally treated with prednisolone (initially 45 mg/day). Laparotomy revealed

**Abbreviations:** AGA, antigliadin antibodies; GFD, gluten free diet; EATL, enteropathy associated T cell lymphoma; EBV, Epstein-Barr virus; EMA, endomysial antibodies; IELs, intraepithelial lymphocytes; LCA, leucocyte common antigen; mAbs, monoclonal antibodies; PCR, polymerase chain reaction; TCR, T cell receptor.

**Table 1** Clinical and genotypic characteristics of patient Nos 1–7

| Patient No/Sex | HLADQB*0201 | Age (y) at primary diagnosis | Age (y) at lymphoma* | Morphology† | Treatment | Monoclonality (TCRγ) | Survival (time from lymphoma diagnosis) |
|---|---|---|---|---|---|---|---|
| 1 (M) | + | 58 | 58, c | Type 3c | Steroids, GFD | Yes‡ | Alive >13 years |
| 2 (M) | + | 69 | 69, c | Type 3a | Surgery, GFD, cyclosporin | Yes‡ | Alive >5 years |
| 3 (F) | + | 69 | 69** | Type 3c | GFD, steroids, Leukeran, CHOP | Yes | Died (1 year) |
| 4 (F) | + | 62 | 62** | Type 3c | Steroids, CHOP | Inconclusive | Died (3 months) |
| 5 (M) | + | 44 | 57, o | Type 3a | GFD, CHOP | Yes | Died (1 year) |
| 6 (M) | nd | 60 | 60, o | Type 3a | Surgery, CHOP | Yes | Died (7 months) |
| 7 (F) | nd | 34 | 57, o | Type 3c | Surgery | Yes | Died (5 months) |

*c, cryptic; o, overt; **intermediate stage between cryptic and overt EATL possible (see text).
†Modified Marsh classification.[13]
‡Present both at diagnosis and 12 or 4.5 years later.
TCR, T cell receptor; GFD, gluten free diet; nd, not determined.

mesenteric cavitation. After temporary total parenteral nutrition, he improved rapidly. Steroids were discontinued after two years and the patient thereafter followed a strict GFD. He is in good health 13 years later despite the persistence of monoclonal IELs (table 1).

### Patient No 2

Patient No 2 had a daughter and a cousin with coeliac disease. He was healthy until the age of 61 years when he suffered intermittent abdominal pain followed by increasing weakness, anorexia, weight loss, diarrhoea, and eventually anasarca over the next five years. Surgery was performed to remove stenotic jejunal lesions. A diagnosis of atypical lymphoid hyperplasia was made on the basis of increased numbers of phenotypically abnormal monoclonal IELs adjacent to large benign appearing ulcers with bacterial overgrowth. The EMA test was negative but AGA titre was elevated. On parenteral nutrition, and later a GFD, the patient improved, but due to persistent monoclonal IELs cyclosporin was additionally instituted for nine months. He later developed an Epstein-Barr virus (EBV) positive intestinal B cell lymphoma which was cured on surgical resection and three CHOP (cyclophosphamide, doxorubicin, vincristin, prednisone) courses (Farstad IN, *et al*, manuscript submitted). He is in good health on a strict GFD five years after the initial diagnosis, despite persistence of monoclonal IELs (table 1).

### Patient No 3

Patient No 3 presented with a clinical picture of coeliac disease, a diagnosis supported by histology and an elevated AGA titre. She responded poorly to a GFD, and one year later jejunal biopsies disclosed a flat mucosa heavily infiltrated with phenotypically abnormal lymphocytes mainly in the epithelium that was diagnosed as low grade T cell lymphoma. Jejunal ulcers were identified radiologically but could not be subjected to biopsy. She was treated with prednisolone (initially 30 mg/day) for one month and then with chlorambucil. Due to lack of clinical response, total parenteral nutrition was instituted for one month before CHOP courses were initiated. The patient died from severe malabsorption six weeks later. Autopsy was not performed.

### Patient No 4

Patient No 4 had a history of gastrointestinal discomfort for many years and was very slim. Two years before her death she experienced long lasting diarrhoea and later weight loss, increasing weakness, and signs of liver failure. Colonoscopy revealed multiple ulcers along the entire large bowel, morphologically diagnosed as part of an unspecific inflammation with granulation tissue and epithelial regeneration. Biopsies from non-involved mucosa were not obtained. The AGA titre was elevated while the EMA test was negative. Jejunal biopsy specimens were oedematous with a flat mucosa harbouring phenotypically abnormal IELs. Analysis for T cell clonality was inconclusive (additional biopsy specimens could not be obtained due to extreme bleeding tendency). The patient had peripheral T cell depletion and oesophageal herpes simplex virus infection as well as systemic opportunistic infection (*Stenotrophomonas maltophilia*). She was tentatively treated with high dose steroids in addition to antibiotics and later with one course of CHOP on the suspicion of T cell lymphoma. At autopsy, heavy mesenteric cavitation and splenic atrophy were found but no further evidence of lymphoma.

### Patient No 5

Patient No 5 was diagnosed as having coeliac disease by histology at the age of 44 years. He was successfully treated with a GFD which was claimed to be strictly followed later on. Twelve years thereafter he experienced abdominal pain, anorexia, and weight loss. Multiple stenotic tumour lesions were removed from the small intestine and were diagnosed as EATL on the basis of morphology and immunohistochemistry. He was treated with three courses of CHOP but died one year later. Autopsy was not performed. Re-examination of the biopsy obtained at the primary diagnosis revealed no sign of neoplastic IELs.

### Patient No 6

Patient No 6 had abdominal discomfort and wasting 1–2 years before the age of 60 years when he experienced sudden severe abdominal pain for which emergency surgery was performed. Stenotic ulcerative tumours were removed from multiple sites along the small intestine and diagnosed as EATL by morphology and immunohistochemistry. He had an elevated AGA titre and a weakly positive EMA test. He was treated with CHOP courses but died seven months after the initial diagnosis.

### Patient No 7

Patient No 7 was diagnosed as having coeliac disease at age 34 years on the basis of morphology and a good clinical response to a GFD. A strict GFD was claimed to be followed later on. For the last 20 years she has also suffered from rheumatoid arthritis but did not receive immunosuppressive drugs. An EMA test performed at age 55 years was negative. At age 57 years she developed abdominal pain and weight loss. A duodenal biopsy was obtained prior to laparotomy on the suspicion of lymphoma, and jejunal resection performed thereafter disclosed multifocal EATL. The patient died five months after this diagnosis.

Patient Nos 1 and 2 were retrospectively deemed to represent variants of cryptic EATL according to recent diagnostic criteria.[2 6]

## Histology and immunohistochemistry

The tissue material used for analysis consisted of jejunal biopsy specimens (patient Nos 1 and 3), jejunal biopsies and

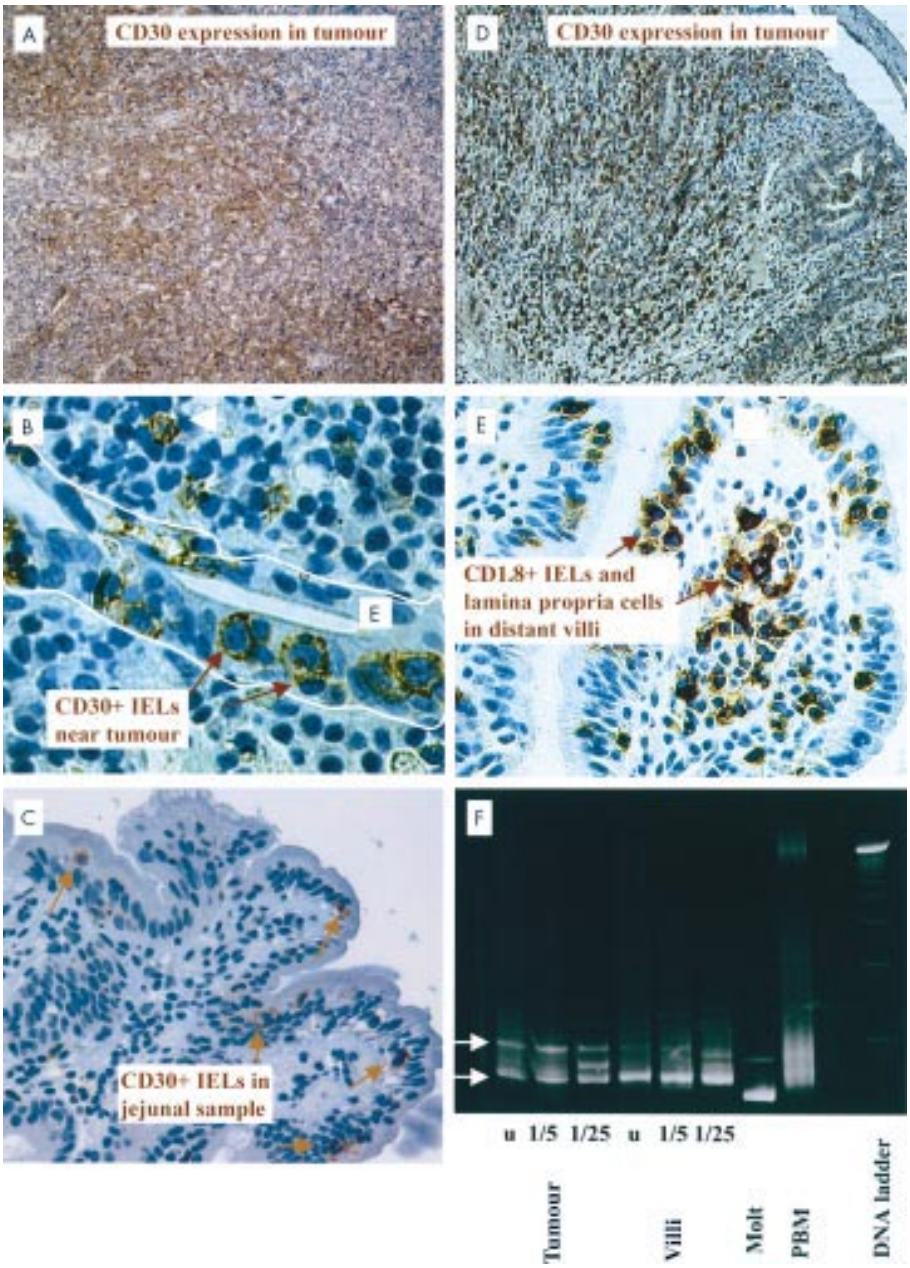


**Figure 1** Immunomorphological and genotypic observations in overt enteropathy associated T cell lymphoma (EATL) (A–C, patient No 5; D–F, patient No 6). (A) Detail of ulcerated tumour with CD30+ tumour cells in a surgical resection specimen (original magnification ×200). (B) Detail of epithelium adjacent to CD30+ tumour in (A) (original magnification ×400). Numerous intraepithelial lymphocytes express CD30 (arrows). (C) Occasional intraepithelial lymphocytes express CD30 (arrows) in a jejunal biopsy specimen (original magnification ×400). (D) Detail of ulcerated tumour with CD30+ tumour cells in a surgical resection specimen (original magnification ×100). (E) Villus a long way away from the tumour showing numerous intraepithelial and lamina propria cells (arrows) expressing CD30 (original magnification ×400). (F) Polymerase chain reaction analysis of DNA extracted from tumour and intact mucosa (villi). Samples were undiluted (u), or diluted 1/5 or 1/25; visible bands (arrows) were present in the same position of all samples, indicating that the same T cell clone was present in villi and in the tumour.

surgical resection specimens (patient Nos 2, 5, and 7), surgical resection specimens only (patient No 6), and jejunal biopsies as well as autopsy (patient No 4). Only formalin fixed material was available from patient Nos 6 and 7, whereas fresh specimens for immunophenotyping were additionally obtained from the others. From patient Nos 1 and 2, biopsy material was also available 12 and five years after the initial diagnosis.

On formalin fixed tissue sections (2–3 μm), immunophenotyping was performed in the Ventana Automated Immunohistochemistry System (Ventana Medical Systems, Tucson, Arizona, USA) after dewaxing and boiling in citrate buffer (pH 6.5) by microwaving (three minutes full effect, 20 minutes 10% effect), and the reaction was developed with the Ventana DAB-kit. The primary monoclonal antibodies (mAbs) were directed to leucocyte common antigen (LCA) (CD45RA/RB, clone 2B11+PD7/26, 1 mg/l; Dako, Glostrup, Denmark), CD3 (ε chain, rabbit IgG, 1.2 mg/l; Dako), CD4 (1F6, culture supernatant 1/5; Novocastra Laboratories Ltd, Newcastle, UK), CD8 (C8/144B, 15 mg/l; Dako), CD30 (BerH2, 49 mg/l; Dako), CD56 (1B6, 1/50; Novocastra), or CD57 (NK-1, 1/100; Novocastra).

Parallel fresh tissue specimens were immediately frozen in liquid nitrogen and prepared for cryosectioning and multicolour immunofluorescence staining as previously described.[12] In

**Table 2** Immunophenotypic characteristics of intraepithelial lymphocytes (IELs) in patients Nos 1–7 compared with IEL phenotypes of normal small intestinal mucosa and coeliac disease (CD) lesions (median and range referring to proportions of CD3+ cells unless otherwise indicated)

| | LCA | CD3ε* | CD8 | CD30† | CD94 | TCRγ/δ | TCRα/β |
|---|---|---|---|---|---|---|---|
| Category | | | | | | | |
| Normal | 100 | 59 (37–81)[a] | 90 (80–95)[b] | 0 (0)[c] | 27 (16–38)[d] | 2 (1–39)[e] | 97 (70–99)[b] |
| CD | 100 | 98 (97–100)[a] | 80 (46–88) | 0 (0–1)[c] | 40§ (29–56)[d] | 20 (11–53)[e] | 80 (78–45) |
| Patients | | | | | | | |
| 1 | 100 | 100 | <1 | 1.6 | 95 | <1 | 100 |
| 2 | 100 | 98‡ | 6 | 0.6 | 27 | 3.5 | 15 |
| 3 | 100 | 99‡ | <0.5 | 0.2 | <5 | 0 | 100 |
| 4 | 100 | 100 | 1 | 25 | 88 | 7 | 15 |
| 5 | 100 | 99 | 15 | 23 | 80 | 1 | 15 |
| 6 | 100 | >90 | 90 | 22 | nd | nd | nd |
| 7 | 100 | >90 | 95 | 18 | nd | nd | nd |

*Proportion of LCA+ (CD45+) cells.
†Proportion of CD30+ cells accumulated for areas near and distant from tumours; near tumors, up to 50% of IELs expressed CD30 (see fig 1C).
‡Additionally examined by flow cytometry where <30% (patient No 2) and <5% (patient No 3) expressed CD3 (Farstad IN, *et al*, unpublished observations).
Data based on: [a]Eiras and colleagues[14]; [b]Brandtzaeg and colleagues[28]; [c]Murray and colleagues[8] (and Farstad IN, *et al*, unpublished observations); [d]Jabri and colleagues[16]; and [e]Halstensen and colleagues.[12]
§Indicated as number of CD94+ cells/100 epithelial cells; by flow cytometry, a median of 70% CD103+ IELs expressed CD94.[16]
LCA, leucocyte common antigen; TCR, T cell receptor; nd, not determined.

brief, mixtures of pretitrated mAbs to LCA (2B11+PD7/26, mouse IgG1), CD3 (ε chain; RIV9, mouse IgG3; Sanbio, the Netherlands), CD4 (SK3, mouse IgG1; BD Biosciences, Erembodegem, Belgium), CD7 (4H9, mouse IgG2a; BD) CD8 (SK1, mouse IgG1; BD), CD30 (BerH2, mouse IgG1), CD94 (HP-3D9, IgG1; PharMingen International, San Diego, La Jolla, California, USA), TCRγδ (5A6.E9 mouse IgG1; HybriDomus, Rhoon, the Netherlands), TCRαβ (8A3, mouse IgG1, recognising the β chain; T Cell Sciences Inc., Cambridge, Massachusetts, USA), and αEβ7 (Ber-ACT8, mouse IgG1; courtesy of H Stein, Berlin, Germany) were followed by Cy3 and FITC conjugated subclass specific goat antimouse IgG (exhibiting red and green fluorescence, respectively) mixed with a rabbit anticytokeratin IgG; a tertiary incubation was with AMCA conjugated antirabbit IgG (exhibiting blue fluorescence). In addition, mAb to HLA-DQ2 (clone 2.12.E11, ascitic fluid diluted 1/5000; courtesy of H Viken, Institute of Transplantation Immunology, Oslo, Norway) was followed by biotinylated horse antimouse IgG (Vector Laboratories, Burlingame, California, USA) and streptavidin-Texas Red (Gibco BRL, Gaithersburg, Maryland, USA) to examine HLA-DQ2 expression in each patient. Proportions of LCA+ cells expressing CD3, CD7, and αEβ7 as well as proportions of CD3+ cells expressing CD4, CD8, CD30, CD94, TCRγδ, or TCRαβ were recorded in a Leitz DMRXE microscope (Leica, Wetzlar, Germany) equipped with filter blocks for observation of red, green, blue, and combined red/green (yellow) emissions. Pictures (fig 1A–E) were obtained with a Spot Camera (Diagnostic Instruments Inc., Brattleboro, USA) mounted on a Nikon Eclipse 800 microscope (Nikon, Tokyo, Japan) and further adjusted with Microsoft Power Point software.

### PCR analysis

T cell clonality was determined by multiplex polymerase chain reaction (PCR) designed to detect all possible rearrangements of the TCRγ gene. Genomic DNA was isolated from three 10 μm thick frozen or formaldehyde fixed sections with the NucleonHT kit (Amersham-Pharmacia Biotech). This DNA (20 ng and 100 ng) was used as a template for the multiplex PCR (primer sequences available on request) and subjected to 35 cycles of amplification with Taq[2000] (Stratagene, La Jolla, California, USA). The products were resolved by electrophoresis on a 5% (w/v) polyacrylamide gel. When multiple samples from the same patient were analysed, they were resolved in adjacent lanes to confirm the same PCR product length in each sample. Peripheral blood mononuclear cells from a healthy individual gave rise to a 30–40 bp broad smear and served as a negative control, while DNA from the Molt T cell line served as a positive control for monoclonality (fig 1F).

## RESULTS

### Morphology and immunohistochemistry

Histological findings in intact mucosa were categorised according to the modified Marsh classification (table 1), as suggested previously.[13] Phenotypes of IELs revealed by multicolour immunofluorescence staining of cryosections (including antiserum to cytokeratin to ensure intraepithelial location of T cells) and by immunoenzyme staining of formalin fixed sections of IELs are summarised in table 2. Phenotypes of IELs were recorded both adjacent to the tumours (when present) and at least 20 villi away from the tumours. Virtually all IELs in these patients expressed both LCA and CD3 (table 2), consistent with a coeliac phenotype,[14] as well as CD7 and αEβ7. All five patients examined expressed the HLADQB*0201 chain known to be associated with coeliac disease (cryosections were not available from patient Nos 6 and 7). The natural killer cell markers CD56 and CD57 were both negative on tumour cells as well as on IELs in all patients (data not shown).

#### Patient No 1

Patient No 1 had a flat mucosa with heavy epithelial lymphocyte infiltration corresponding to Marsh 3c at the time of diagnosis. These IELs expressed CD3, TCRαβ, and the natural killer receptor CD94 but were mainly negative for CD4, CD8, and CD30 (table 2). On re-examination 12 years later, immunohistology revealed a Marsh 3a lesion with persistence of a predominant population of CD3+CD8-CD4-TCRαβ+ cells, although 37% of CD3+ cells then expressed CD8 (data not shown).

#### Patient No 2

Initial jejunal biopsies showed slight villous atrophy and increased IELs compatible with a Marsh 3a lesion. Subsequent jejunal surgical resection specimens revealed numerous large benign appearing ulcers intervening between intact mucosa with Marsh 3a changes. IELs near and distant from the ulcers were phenotypically similar and expressed CD3 but generally not CD4, CD8, TCR, or CD30; however, CD94 was present on approximately one third of IELs (table 2). Sheets of phenotypically abnormal lymphocytes were often seen in villi

adjacent to the ulcers. Occasional CD30+ blast-like cells but no distinct tumours were seen in four of six ulcers. Multiple follow up biopsy specimens have shown IELs with a similar phenotype.

### Patient No 3

Multiple (n=8) jejunal biopsy specimens showed a flat mucosa with heavy lymphocyte infiltration in the epithelium and in the lamina propria, compatible with a Marsh 3c lesion. These lymphocytes were slightly enlarged with more pale nuclei than ordinary IELs. They expressed CD3 and TCRαβ but were virtually negative for CD4, CD8, CD94, and CD30 (table 2).

### Patient No 4

Jejunal biopsy specimens were oedematous with a flattened surface and only slightly increased IELs, compatible with a Marsh 3c lesion. IELs expressed CD3 but were mainly negative for TCR, CD4, and CD8 but positive for CD94; however, a large fraction (25%) expressed CD30 (table 2). Occasional clusters of 5–10 such CD30+ cells were also seen in the lamina propria, but without morphologically identifiable tumours, and no ulcers were found.

### Patient No 5

Jejunal surgical resection specimens showed multiple ulcers overlying CD30+ tumours of variable size (fig 1A). The tumour cells were also positive for CD3 but negative for CD4 and CD8. Intervening mucosa was intact with variable villous atrophy and increased IELs, compatible with a Marsh 3a lesion. IELs were phenotypically similar near and distant from tumours in that they expressed CD3 but generally no CD4 or CD8. (table 2). Again, sheets of phenotypically abnormal lymphocytes were found in villi adjacent to tumours. In such areas, many IELs and some lamina propria cells expressed CD30, often merging with tumour tissue (fig 1B). Jejunal biopsy specimens obtained eight months after diagnosis of EATL showed a Marsh 3a lesion with similar IELs that were mainly negative for TCR but usually positive for CD94, and some blast-like IELs also positive for CD30 (23%; fig 1C, table 2). Re-examination of the biopsy specimen obtained at primary diagnosis showed a Marsh 3c lesion with predominant CD3+CD8+CD4– IELs (TCR and CD94 could not be investigated on this formalin fixed material).

### Patient No 6

Jejunal resection specimens revealed multiple CD30+ tumours with ulcerated mucosa (fig 1D). The tumour cells also expressed CD3 and CD8 but not CD4. Intervening intact mucosa disclosed Marsh 3a lesions. IELs in intact mucosa were also positive for CD8 (table 2) but mainly negative for CD4. Because fresh tissue was unavailable, examination of TCR and CD94 could not be performed. Blast-like IELs and lamina propria cells occurring both a long way away from (at least 20 villi; fig 1E) and close to tumours expressed CD30.

### Patient No 7

The jejunal resection specimen contained multiple stenotic ulcers intervening between intact mucosa. Histology revealed multifocal EATL. The tumour cells expressed CD3, weakly CD8, and CD30. Intervening mucosa was mostly flat, corresponding to a Marsh 3c lesion. Blast-like IELs appearing both near tumours and in clusters a very long way away (many mucosal folds) had a similar phenotypic profile. CD4 was not expressed by tumour cells and generally not by IELs. In a duodenal biopsy specimen obtained one week prior to operation, a Marsh 3b lesion was present with IELs expressing CD3, weak or strong CD8 and, at one site, many blast-like cells with CD30. Fresh tissue for examination of CD94 and TCR was unavailable from this patient.

### PCR analysis

A monoclonal T cell population was found in all patients except for patient No 4 for whom data were inconclusive (table 1). Although IELs are reported to be oligoclonal,[15] it has not been difficult to differentiate these patients from most ordinary coeliac disease patients and controls in our material because the latter two have had polyclonal patterns of the TCRγ gene in intestinal biopsy specimens (data not shown). In patient No 1, a monoclonal T cell population was detected at the time of diagnosis as well as in a control biopsy obtained 12 years later. In patient No 2, monoclonality for TCR was detected in multiple biopsy specimens, the latest obtained five years after the initial diagnosis, as well as in surgical resection specimens from ulcers and intact mucosa containing phenotypically abnormal IELs (data not shown). A monoclonal T cell population was detected both in tumours and in intact mucosa from patient Nos 5, 6, and 7, as well as in biopsy specimens obtained eight months after lymphoma diagnosis in patient No 5. The biopsy specimen obtained at primary diagnosis in patient No 5 showed no monoclonal T cell population. Where multiple sites within a patient were examined simultaneously, the PCR products revealed bands at a similar position (patient Nos 2, 5, 6, and 7; fig 1F).

## DISCUSSION

Progression to overt lymphoma in patients with refractory coeliac disease is difficult to predict even when the neoplastic nature of IELs has been established.[2] Although overt EATL development seems to be a rare event, it is important to identify potentially curable stages of this lymphoma. We have reported here that in three patients with overt EATL, CD30 was expressed on IELs adjacent to or merging with overt tumours as well as on scattered IELs in villi a long way away from such tumours and even in jejunal biopsy specimens (fig 1B, 1C, 1E). In contrast, two other patients who had phenotypically abnormal and monoclonal IELs but no tumours and extremely few CD30+ cells are both alive many years after the initial diagnosis. One patient who died shortly after diagnosis had CD30+ IELs at presentation, and one patient whose IELs did not express CD30 died 1.5 years after diagnosis. No consistent pattern of IEL phenotypes, T cell clonality, or morphological features was found in relation to prognosis in these patients. However, with the exception of patient No 3 who had very few CD30+ cells in the investigated part of her mucosa (ulcers unavailable for examination), CD30 expression by IELs appeared to indicate a worse prognosis, and in three patients the presence of overt EATL.

The patients described here had more heterogeneous IEL phenotypes than previously reported for similar cases.[3] With the βF1 antibody detecting the β chain of the TCRαβ, both patient Nos 1 and 3 had retained β chain expression but differed markedly in CD94 expression (table 2). IELs of patient No 1 were found to express mainly the β11 chain variant (90%; Halstensen TS, Brenner MB, van Kerckhove C, unpublished observations) but this was not examined for patient No 3. CD94 was expressed in the majority of IELs in patient Nos 1, 4, and 5 (table 2), in agreement with the findings reported in ordinary coeliac disease[16] (Farstad IN, *et al*, unpublished observations). Patient Nos 2 and 3, who both had ulcers and low levels of CD94, differed in TCR expression, as revealed by immunohistochemistry (table 2) although surface CD3 was lacking on IELs from both by flow cytometric analysis (Farstad IN, *et al*, unpublished observations), the latter in agreement with recent data.[2 3] In patient Nos 6 and 7, both IELs and tumour cells expressed CD8 but not CD56 (or CD57). IELs expressing TCRαβ, TCRγδ, or ordinary strong CD8 in patient Nos 2, 4, 5 and 7 (table 2) presumably represented remnants of the normal IEL population.

One may question to what extent our patients were coeliac disease cases. Only patients with overt EATL could strictly be

diagnosed as having coeliac disease—patient Nos 5 and 7 because of a satisfactory response to a GFD for several years, and patient No 6 on the basis of a positive EMA test. The negative EMA test in patient No 7 two years prior to lymphoma diagnosis may reflect the fact that she followed a strict GFD. Patient No 2 was EMA negative and patient Nos 1 and 3 were diagnosed before this test was available. However, these patients were HLA-DQ2 positive (table 1) and two (patient Nos 1 and 2) are in good health on a GFD several years after the initial diagnosis. Patient No 3 had a Marsh 3c lesion and elevated AGA. The exact nature of her condition is unknown but available data favour a diagnosis of primary refractory coeliac disease. Patient No 4 most likely represented a case of longstanding unrecognised coeliac disease based on clinical and morphological findings (table 1); despite the lack of EMA, AGA titre was elevated and she developed severe malabsorption with serious complications prior to diagnosis. In common with patient No 1, she had mesenteric cavitation and in addition splenic atrophy that are both associated with refractory coeliac disease.[2 3] Autoimmune enteropathy was excluded on the basis that all of our patients had elevated numbers of IELs, a feature which is not reported in this condition.

Whether CD30 expression might be pathogenetically related to lymphoma development is not known. CD30 is expressed by activated T cells, EBV infected cells, Reed-Sternberg cells in Hodgkin's lymphoma, and in several non-Hodgkin's T and B cell lymphomas, including EATL,[4 17] but the functional implication of this marker on neoplastic cells remains elusive. CD30 has been postulated to mediate signalling for cytokine production, especially for T helper 2 cytokines.[17 18] Adding to the confusion of CD30 expression in lymphomas, lymphoproliferative disorders of the skin may have a relatively good prognosis when expressing CD30[19 20] whereas that of EATL (usually CD30+) is poor. In agreement with Murray and colleagues,[8] we found that normal intestinal mucosa contains quite rare CD30+ cells, except in solitary lymphoid aggregates and Peyer's patches where some cells in the interfollicular zones expressed CD30 (Farstad IN, unpublished observations), as reported for tonsils and activated lymph nodes.[17] Bamford and colleagues[21] found very few gastric T cells expressing CD30 although colonic counterparts frequently were CD30+.

Our patient Nos 1 and 2 had virtually no CD30+ cells at presentation or after 12 and five years of follow up whereas patient Nos 5, 6, and 7 (who died) had CD30+ IELs a long way away from the CD30+ tumours when lymphoma was diagnosed (fig 1B, 1C). Importantly, CD30+ IELs were also present in jejunal biopsy specimens from patient Nos 5 (fig 1C) and 7 (data not shown). In the latest biopsy specimen obtained from patient No 2 however, occasional IELs and lamina propria cells expressed CD30 (data not shown) although no ulcers were found. Alternative treatment is now being considered for him. Patient No 4 had many IELs expressing CD30 in the absence of both ulcers and distinct tumours (table 2) although clusters of CD30+ cells were occasionally seen in the lamina propria. It is possible that her lesion represented an intermediate stage of lymphoma development. The exact nature of the lymphoma in patient No 3 is not known, both because ulcers were unavailable for examination and because no autopsy was performed. Benign appearing ulcers can precede the development of overt EATL,[5] and occasional CD30+ cells were found in ulcers from our patient No 2 at initial diagnosis. Theoretically, CD30 expression by IELs and/or lamina propria cells may precede ulcer formation. However, tumours may develop directly from CD30+ cells in the lamina propria, as possibly suggested by data from patient Nos 6 and 7 where clusters of CD30+ IELs and lamina propria cells were observed a long way away from overt tumours (fig 1E).

Previous data have indicated that the coeliac pathogenesis is orchestrated by gluten specific CD4+ lamina propria T cells.[22] Although these CD4+ T cells are activated (CD25+) in untreated coeliac disease and after gluten challenge ex vivo, they do not proliferate in situ.[23] CD8+TCRαβ+ IELs are both markedly increased in number and proliferate in the untreated coeliac lesion[16 23] whereas they subside on gluten withdrawal.[24] Interleukin 15 may be secreted by epithelial cells[25] and is a potent inducer of IEL proliferation[26]; it also seems to have a role in the manifestations of coeliac disease through a direct effect of gluten on the epithelium.[27] It is likely that in some patients with refractory or untreated disease, growth factors released by gluten responsive cells, perhaps including epithelial interleukin 15, induce proliferation of CD8+ IELs that in turn become more prone to spontaneous mutations and malignant development.

Our data are in line with previous reports[5–8] suggesting that the same monoclonal T cell population can be detected in intact mucosa, in benign appearing ulcers, and in overt tumours from the same patient. As also indicated in those reports, the neoplastic IELs were found at multiple intestinal sites in the same patient, supporting the notion that cryptic EATL in principle affects the entire length of the small intestine. The observed CD30 expression by IELs found in patients with CD30+ tumours adds to the idea that overt EATL also originates from IELs. That such CD30+ IELs were observed a long way away from overt tumours and even in jejunal biopsy specimens suggests that CD30 should be included in the screening for malignant development in patients with cryptic EATL.

## ACKNOWLEDGEMENTS

The following colleagues are gratefully acknowledged for contributing tissue specimens and clinical information regarding patient No 5: Drs May-Bente Bengtsson, Vestfold Central Hospital, and Ida Ikonomou, Norwegian Radium Hospital. Mr Hogne Røed Nilsen, Department of Pathology, Rikshospitalet, is greatfully acknowledged for valuable technical assistance, and Dr Knut EA Lundin, Department of Medicine, Rikshospitalet, for critical reading of the manuscript.

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