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Numbers of T cell receptor (TCR) αβ+ but not of TcR γδ+ intraepithelial lymphocytes correlate with the grade of villous atrophy in coeliac patients on a long term normal diet

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Abstract

Numbers of T cell receptor (TcR) γδ+ and αβ+ intestinal lymphocytes were studied in 34 coeliac patients in respect of their diet and the grade of villous atrophy. Particular attention was given to a group of 21 patients with coeliac disease according to ESPGAN criteria who were on a well tolerated long term normal diet and in nine of whom the muscosa had returned to normal or nearly normal. A significant increase in TcR γδ+ cells was observed in the gut epithelium of coeliac patients compared with age matched controls, and this did not correlate with either the presence of gluten in the diet or with the grade of villous atrophy. Thus, numbers of TcR γδ+ intraepithelial lymphocytes (IEL) were considerably above the normal range in four of seven patients on a gluten free diet and in four of nine patients who had recovered a normal or nearly normal mucosa in spite of a normal diet. In contrast, numbers of intestinal TcR αβ+ cells varied with the stage of the disease. Their number was high in the epithelium of patients with active coeliac disease (n=18) but significantly less in patients whose mucosa had returned to normal or nearly normal either after gluten free diet (n=7) or in spite of a normal diet (n=9). Immunohistochemical markers of intestinal mononuclear cell activation detected in active coeliac disease were either weakly expressed or absent in the latter patients. It is suggested that TcR αβ+ but not TcR γδ+ IEL are sensitised to gliadin in coeliac disease, and that only the former cells play a direct part in the pathogenesis of the villous atrophy. The normal counts of TcR αβ+ IEL and the absence of detectable mononuclear activation in the biopsy specimens of a few patients who have recovered clinical and histological tolerance to gluten sustains this hypothesis and also suggests that immunological tolerance to gluten may be acquired in a subgroup of coeliac patients. The appreciable increase in TcR γδ+ IEL observed in some of the latter patients, however, is similar to that observed in latent coeliac disease urging for their careful and prolonged follow up until the role of TcR γδ+ IEL in the pathogenesis of coeliac disease is elucidated.

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In coeliac disease several observations suggest that intestinal lymphocytes, and particularly intraepithelial lymphocytes (IEL), play a part in the development of villous atrophy. Thus, epithelial lesions in coeliac disease resemble those induced by activated intestinal T cells in experimental graft *versus* host disease[1 2] or in organotypic culture of fetal intestine.[3] In patients left on a gluten free diet, intraduodenal challenge by gluten induces a dose dependent increase in the numbers of IEL, associated, at the highest doses of gluten, with epithelial changes.[4] In a rat model of gliadin induced enteropathy, IEL from sensitised animals were able to induce epithelial changes when introduced into jejunal loops of germ free animals.[5]

Several recent studies have emphasised the striking increase in IEL bearing a T cell receptor for antigen (TcR) γδ+ in coeliac disease. These cells, present in small numbers in normal human gut epithelium, were much more numerous in coeliac disease patients with active disease or on a gluten free diet.[6–8] Two main subsets of TcR γδ+ cells have been described in humans. One contains a variable δ region encoded by the Vδ1 gene segment. The other subset possesses variable regions encoded by the Vδ2 and Vγ9 gene segments. Both subsets can be identified using monoclonal antibodies (mAb) specific for the encoded proteins.[9] Using these mAb, it has been shown that only Vδ1+ cells were considerably increased in the epithelium of coeliac patients.[6–8] However, the role of TcR γδ+ IEL in the pathogenesis of coeliac disease remains a matter of debate in so far as the functions of TcR γδ+ cells, the nature of the antigens which they recognise, and the mechanisms which drive their selection and expansion, are not elucidated.[9] In contrast with TcR γδ+ cells, intestinal TcR αβ+ cells have raised little interest in coeliac disease. Yet, TcR αβ+ bearing cells form the vast majority of cells in normal gut mucosa. Furthermore, their excessive activation in graft *versus* host disease,[2 10] and probably in certain autoimmune diarrhoeas[11] leads to severe mucosal damage and to villous atrophy.

To investigate further the respective contributions of TcR γδ+ and TCR αβ+ intestinal lymphocytes in the pathogenesis of coeliac disease, we have studied variations in their numbers, depending on the diet and the grade of villous atrophy, in 34 coeliac patients and in 30 adult and paediatric controls. Particular attention was given to the group of coeliac patients left for several years on a clinically and biologically well tolerated normal diet. Indeed, previous observations suggest that a significant proportion of the latter patients may recover a normal or partially normal mucosa despite gluten in the

TABLE I *Description of patients with coeliac disease (CD)*

Disease stage	*No*	*Age (yr)*	*Intestinal histology*
Group A=onset of CD	5	1–10	TVA
Group B=CD on GFD for 1–11 y	7	3–16	2/7 moderate VA 5/7 normal mucosa
Group C=CD according to ESPGAN on a normal diet for:			
1 m to 3 y	8	7 aged 5–15 1 aged 21	STVA/TVA Normal mucosa
3 to 8 y	9	5 aged 5–8 4 aged 9–21	STVA/TVA Normal mucosa* or moderate VA
>10 y	5	1 aged 38 4 aged 15–30	TVA Normal mucosa or moderate VA

TVA=total villous atrophy, VA=villous atrophy, STVA=subtotal villous atrophy.
* This 21 year old man had had dermatitis herpetiformis diagnosed simultaneously with coeliac disease when he was 12 years. He was free of symptoms at the time of examination.

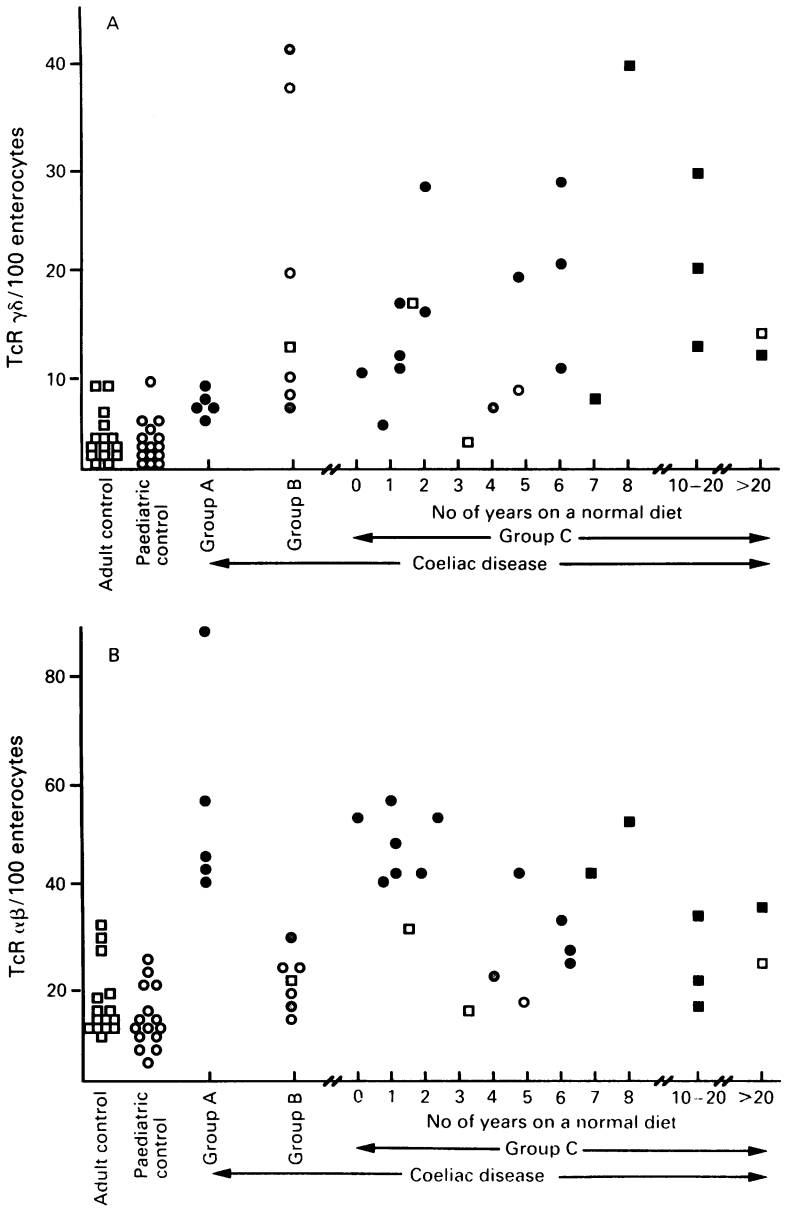

Figure 1: Variations in the surface epithelium of the numbers of T cell receptor (TcR) γδ+ intraepithelial lymphocytes (IEL) (A) and of TcR αβ+ IEL (B) in recently diagnosed coeliac disease patients (group A), patients on a gluten free diet (group B), and in patients with coeliac disease according to ESPGAN left on a normal diet for 1 month to over 20 years (group C). Numbers in adult controls (AC) and in paediatric controls (PC) are indicated. To compare the values in patients and controls according to age, cell counts in children (7 months to 14 years) are indicated with circles and in adults with squares. Closed symbols (● or ■) indicate values in patients with total or subtotal villous atrophy, hatched symbols (◍ or ▩) indicate values in patients with partial villous atrophy, and open symbols (○ or □) indicate values in controls and in patients with a normal mucosa. In (A) numbers of TcR γδ+ IEL/100EC varied from 0·2 to 9 in adult controls and from 0·4 to 10 in paediatric controls, the highest value being observed in a 2 year old child with cow's milk intolerance and severe atopic dermatitis. In B, numbers of TcR αβ+ IEL/100EC varied from 11 to 33 in adult controls, from 3 to 21 in paediatric controls aged 7 months to 2 years and from 8 to 27 in older paediatric controls.

diet. Thus, among 29 coeliac patients followed in our unit and left long term on a well tolerated normal diet, four recovered a normal mucosa and six a partially normal mucosa.[12 13] Similarly, in a long term analysis of relapses, Shmerling and Franckx observed that six of 88 patients (7%) failed to relapse after a two year challenge and 11 (13%) showed only a partial villous atrophy.[14] Finally, Mäki *et al* observed that four of 38 coeliac patients (11%) undergoing a postpubertal gluten challenge did not relapse within two years and four (11%) showed only moderate villous atrophy.[15] The present study aimed to define which changes in intestinal T cell subsets accompanied spontaneous partial or total mucosal recovery.

Patients and controls

PATIENTS

Thirty four coeliac patients aged between 1 and 38 years of age were studied (Table I) The groups were as follows:

Five children studied at the time of diagnosis presented with a typical malabsorption syndrome and total villous atrophy. The diagnosis of coeliac disease was subsequently ascertained by the beneficial effect of gluten free diet on diarrhoea and growth (group A).

Seven patients studied after 1–11 years of a strict gluten free diet had recovered a normal (n=5) or nearly normal mucosa (group B).

Twenty two patients with coeliac disease according to ESPGAN criteria[16 17] were left for 1 month to over 10 years on a normal diet according to a protocol designed several years ago[13 14] (group C). Close follow up indicated that gluten was well tolerated both clinically (with regard to digestive symptoms and growth) and biologically in all patients except one, a 6 year old boy who presented with severe clinical relapse after a one month of gluten challenge and was therefore put back on a gluten free diet. Intestinal biopsies were performed in the course of the regular follow up of these patients. Their results are shown in Table I. Thirteen patients in group C had total or subtotal villous atrophy, five patients had moderate villous atrophy (ratio of villus height/crypt depth >1), 4 had recovered a normal mucosa. As previously reported,[12 13] the longer the time spent on a normal diet, the greater was the number of patients with moderate villous atrophy or normal mucosa. Thus only one of eight patients on a normal diet for less than 3 years had recovered a normal mucosa. After 3–8 years on a normal diet, four of nine patients had normal or nearly normal mucosa. After 10 years, four of five patients had recovered a normal or nearly normal mucosa.

In nine patients at various stages of coeliac disease, peripheral blood was obtained on the day of the intestinal biopsies.

CONTROL SUBJECTS

Normal control intestinal biopsy specimens were obtained from 15 children. Nine had short stature, one cow's milk tolerance, three non-specific chronic diarrhoea, one prolonged diar-

rhoea after salmonella infection and one had short bowel syndrome after extensive intestinal resection. Specimens were also taken from 15 adults undergoing intestinal surgery for gastric and pancreatic benign or malignant diseases.

Methods

MONOCLONAL ANTIBODIES

Monoclonal antibodies (mAb) were directed to CD3 (Leu4), CD4 (Leu3a), CD8 (Leu2a), CD25, HLA-DR non-polymorphic determinants (Becton Dickinson, Grenoble, France), to the framework of the TcR αß (ßF1, gift from Dr M Brenner and BMA031 from Behring (Rueil Malmaison, France)), to the framework of the TcR γδ (TcRδ1, gift from Dr M Brenner). Subsets of TcR γδ+ cells were studied using monoclonal antibodies anti-TiγA, anti-TiVδ2 (gifts from Dr T Hercend) and δTCS1 (T Cell Sciences, distributed by Amersham, Versailles, France). These antibodies respectively react with Vγ9, Vδ2, and Vδ1 encoded TcR.[9]

IMMUNOHISTOCHEMICAL STUDIES

All biopsies were performed with a paediatric Watson capsule and specimens were frozen in liquid nitrogen. Cryostat sections were either stained with haematein and eosin to score the grade of villous atrophy or labelled using previously described simple or double immunoperoxidase techniques.[18 19] Results of immunostaining were expressed either quantitatively or semiquantitatively. Numbers of labelled IEL were estimated by counting the peroxidase stained cells per 100 epithelial cells, 500 to 1000 enterocytes being counted for each mAb in the surface and in the crypt epithelium respectively. Numbers of labelled lymphocytes in the upper and pericryptic lamina propria were counted per field at a ×250 magnification. Statistical analysis was performed using a Student's *t* test; p values less than 0·05 were considered as significant. CD25 positive cells were recorded as numerous, rare, or absent. Expression of HLA-DR by enterocytes was noted as absent, weak, or strong.

STUDY OF PERIPHERAL BLOOD TcR γδ+ CELL SUBSETS

Ficoll-Hypaque separation of lymphocytes from heparinised blood and membrane immunofluorescence staining with anti-T cell mAb were performed as described.[20] Stained cells were analysed with a cytofluorograph (FACSTAR PLUS, Becton-Dickinson).

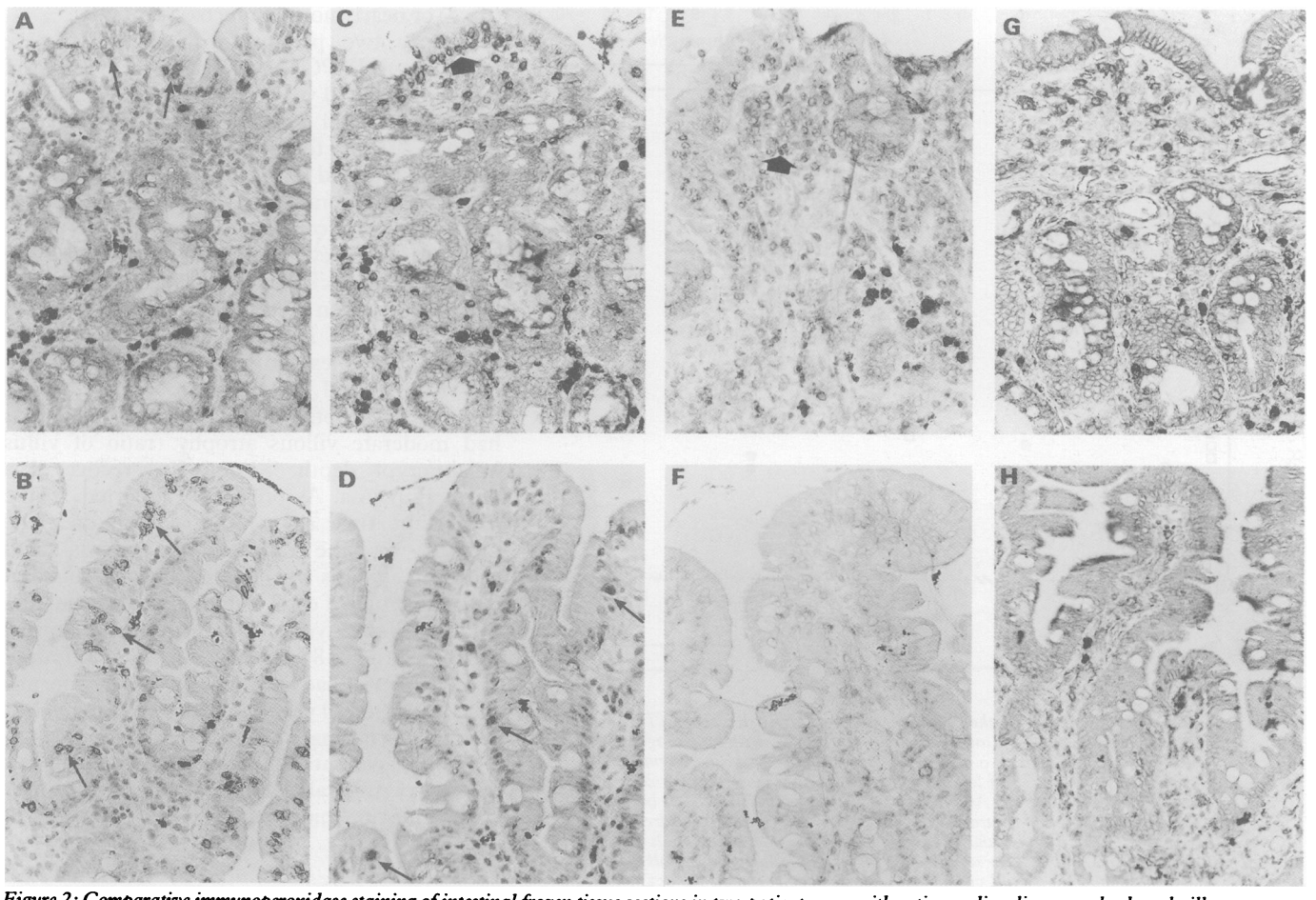

Figure 2: Comparative immunoperoxidase staining of intestinal frozen tissue sections in two patients, one with active coeliac disease and subtotal villous atrophy (A, C, E, and G), the second on a gluten free diet and with a nearly normal mucosa (B, D, F and H). TcR γδ+ cells are numerous in the surface epithelium of both patients (A, B arrows). In contrast, TcR αβ+ cells, numerous in the surface epithelium of the patient with active coeliac disease (C, large arrow), are rare or absent in the patient on a gluten free diet (thin arrows) (D). In the former patient, a large group of CD25+ cells with weak membrane staining are visible in the subepithelial lamina propria (E, large arrow) and anti-HLA-DR antigens are intensely expressed by both surface and crypt enterocytes (G). In the second patient, there is no CD25+ cells (F); HLA-DR expression, intense on villous enterocytes, is weak or absent in the crypts (H).

Results

TcR γδ+ LYMPHOCYTES

The number of TcR γδ+ cells was significantly increased in the epithelium of coeliac patients compared with age matched controls (Figs 1A, 2A and 2B, and Table II). Yet, some overlapping was observed because of wide individual variations in the number of TcR γδ+ IEL both in coeliac disease patients and in controls (Fig 1A, Table II). No changes could be shown in the numbers of TcR γδ+ IEL according to the stage of the disease. There was no correlation with the grade of villous atrophy (Table II, Figs 2A and B) or with the diet (Fig 1A). Thus, TcR γδ+ IEL were high in patients on a gluten free diet (Figs 1A and 2B). Numbers of TcR γδ+ IEL were also considerably above the normal range in five out of the nine patients who had recovered a normal (n=2) or nearly normal mucosa (n=3) (Fig 1A) in spite of a gluten containing diet. In only one coeliac patient were TcR γδ+ IEL values in the low range of control values. This patient had been diagnosed as having dermatitis herpetiformis and coeliac disease at the age of 12 years. When aged 21 years and after three years on a normal diet he presented with a normal mucosa and a normal skin.

The use of antibodies directed to subsets of TcR γδ+ cells allowed us to show that the increase in TcR γδ+ IEL interested Vδ1+ cells and a subset of TcR γδ+ cells which stained with neither anti-Vδ1, nor with anti-Vδ2 and anti-Vγ9 antibodies (Table III). The presence of this unusual third subset of TcR γδ+ cells was confirmed in four coeliac patients by double staining experiments (Fig 3). Similar cells were not readily detected in the epithelium of adult controls.[20]

In order to define whether the observed changes were restricted to the gut epithelium, TcR γδ+ cells were also studied in the lamina propria. A moderate increase in TcR γδ+ cells was detected in the superficial lamina propria but this was significant only when compared with paediatric controls. No increase in TcR γδ+ cells was observed in the pericryptic lamina propria (Table II). In nine patients, numbers and subsets of TcR γδ+ cells were also compared in the blood and gut epithelium. Percentages of TcR γδ+ cells/100 CD3+ cells in the peripheral blood were comparable with those previously reported in normal individuals and were thus lower than in the gut epithelium (=0·011 in paired Student's *t* test). In addition, in all tested patients, the distribution of TcR γδ cells subsets in blood was similar to that observed in normal individuals[9] and differed from the distribution observed in the autologous gut epithilium (Table IV).

TcR αß+ LYMPHOCYTES

In contrast with TcR γδ+ IEL, numbers of TcR αβ+ IEL varied strikingly with the stage of coeliac disease (Table V, Figs 1B, 2C, and 2D). Counts of TcR αβ+ IEL were significantly higher in patients with active coeliac disease and total or subtotal atrophy than in controls, in patients with partial villous atrophy, or in those with a normal mucosa (Table V). Furthermore, all patients in whom the mucosa had returned to normal, whether on a gluten free diet (n=5) or in spite of a normal diet (n=4), had counts of TcR αβ+ IEL comparable with those of age matched controls (Figs 1B, 2D and Table V). Similar variations in the number of TcR αβ+ cells were observed in the superficial lamina propria but were much less obvious. No significant changes were detected in the pericryptic lamina propria (Table V).

When comparing the numbers of TcR αβ+ cells with those of CD4+ or CD8+ cells, it was possible to deduce that most TcR αβ+ IEL were CD8+, as expected. CD4+ IEL, which form a minor subset of the TcR αβ IEL in the normal small intestine[20] were moderately increased in coeliac disease with total or subtotal villous atrophy but this was only marginally significant (Table VI).

Previous studies have indicated that abnormal activation of intestinal T lymphocytes is accompanied by the appearance of CD25+ cells and results in increased expression of HLA-DR antigens by enterocytes.[11] These two immunohistochemical markers of intestinal mononuclear cell activation varied in parallel with the numbers of TcR αβ+ cells. Thus CD25+ large cells resembling macrophages were numerous in the lamina propria of patients with total or subtotal villous atrophy and with raised numbers of TcR

TABLE II *Numbers of T cell receptor (TCR) δ1+ lymphocytes in epithelium and lamina propria (LP) according to the degree of villous atrophy (values mean (SD))*

	No of TCRδ1+ IEL/100 EC		*No of TCRδ1 lymphocytes/field*	
	Surface epithelium	*Crypt epithelium*	*Upper LP*	*Pericryptic LP*
Coeliac disease patients:*				
STVA/TVA (n=18)	14·8 (9·4)†	2·4 (1·9)‡	2·2 (1·8)§	1·0 (1·3)**
Moderate VA (n=7)	17·5 (13·5)†	3·1 (2·0)†	2·8 (2·4)§	1·2 (0·7)π
Normal mucosa (n=9)	14·5 (10·4)†	2·5 (1·7)†	1·7 (1·4)π	0·8 (0·7)**
Pediatric controls (n=15)	3·1 (2·4)	0·6 (0·5)	0·9 (0·4)	0·6 (0·5)
Adult controls (n=15)	3·6 (2·7)	1·1 (0·2)	2·1 (1·5)††	2·1 (1·2)††

* In this table, results were considered according to the grade of villous atrophy only. Among patients with subtotal villous atrophy (STVA) or total villous atrophy (TVA), 5 belonged to group A of recently diagnosed patients, 13 were patients with CD according to ESPGAN criteria left on a normal diet (group C). Among CD patients with moderate villous atrophy (VA), 2 were on GFD (group B of patients), 5 were on a normal diet (group C). Among CD patients with normal mucosa, 5 were on GFD (group B) and 4 on a normal diet (group C).
† $p<0·01$ compared with controls; ‡ $p<0·01$ compared with paediatric controls, $p<0·02$ compared with adult controls; § $p<0·01$ compared with paediatric controls, not significant compared with adult controls; π $p<0·05$ compared with paediatric controls, not significant compared with adult controls; ** not significant compared with controls; †† counts performed in 8 of the 15 adult controls.

TABLE III *Subsets of T cell receptor (TCR) γδ+ cells in the surface epithelium (values mean (SD))*

	No of IEL/100 EC stained with:				*No of TCRδ1+IEL that did not stain with Anti-TiγA, -TiVδ2, or δTCS1**
	TCRδ1	*Anti-TiγA*	*Anti-Ti-Vδ2*	*δTCS1*	
Coeliac disease patients	15·4 (10·3)† n=34	3·1 (2·2)‡ n=33	2·8 (2·0)‡ n=30	6·0 (5·1)§ n=33	5·4 (4·5)π n=33
Paediatric controls	3·1 (2·5) n=15	1·0 (1·1) n=11	0·6 (0·7) n=11	1·0 (1·5) n=14	1·1 (0·9) n=14
Adult controls	3·6 (2·7) n=15	1·7 (2·4) n=10	1·7 (2·3) n=7	1·7 (2·6) n=6	1·7 (0·5) n=6

* Mean of the numbers of (TCRδ1+ IEL)-(anti-TIγA+ or anti-TiVδ2+ IEL)-(δTCS1+ IEL) calculated in each individual.
† $p<0·001$ compared with controls; ‡ $p<0·01$ compared with paediatric controls, not significant compared with adult controls; § $p<0·01$ compared with paediatric controls, $p<0·05$ compared with adult controls; π $p<0·01$ compared with paediatric and adult controls.

TABLE IV *Comparison between percentages and subsets of T cell receptor (TCR) γδ+ cells in the gut epithelium and in the peripheral blood of nine patients with coeliac disease*

	Intraepithelial lymphocytes			*Peripheral blood lymphocytes*		
*Patient no**	*%TcRδ1/ CD3+*	*%TγA or TiVδ2+/ TcRδ1+*	*%δTCS1+/ TcRδ1+*	*%TcRδ1+/ CD3+*	*%TγA or TiVδ2/ TcRδ1+*	*%δTCS1+/ TcRδ1+*
1	7	30	30	10	90	ND
2	10	6	49	6	66	17
3	12	22	25	5	ND	ND
4	42	12	ND	3	50	30
5	32	37	10	13	85	15
6	12	35	46	5	95	0
7	24	10	36	2	95	5
8	56	15	45	6	62	27
9	52	20	50	9	95	5

* Patients 1–3 were just diagnosed with coeliac disease and had total villous atrophy (group A). Patients 4–9 had coeliac disease according to ESPGAN criteria and were on a normal diet (group C). Patient 4 had subtotal villous atrophy; patients 5–9 had moderate villous atrophy or normal mucosa.
ND=not done.

αβ+ IEL. Two patients also had a small number of CD25+ IEL. In these patients, HLA-DR expression was noticeably increased as reflected by the strong labelling of crypt enterocytes. In contrast, in controls as well as in five of seven patients on a gluten free diet and in six of nine patients on a normal diet who had recovered normal or nearly normal counts of TcR αβ+ IEL and a normal mucosa, CD25+ cells were absent (except for a few cells in lymphoid follicles); expression of HLA-DR antigens by crypt enterocytes was undetectable or weak. In the five remaining patients with moderate villous atrophy (two of seven patients on gluten free diet, three of nine coeliac patients on a long term normal diet), rare lamina propria CD25+ cells were noted and HLA-DR expression remained increased on the crypt epithelium.

Discussion

Previous studies have emphasised the possible role of intestinal IEL in the pathogenesis of coeliac disease (see above). The present work, showing striking changes in intraepithelial T cell subsets but little or no changes in lamina propria T cells, sustains this hypothesis. Recently, an increase in gut TcR γδ+ IEL using a Vδ1 gene segment was observed in coeliac patients with active disease or on a gluten free diet[6-8] but not in patients with other diseases associated with villous atrophy.[6 8 11] The present study confirms and extends these results. Firstly, we show the presence of a third subset of TcR γδ+ IEL not found in the normal intestinal mucosa. These IEL may use the Vδ3 gene segment as suggested by studies in molecular biology which identified Vδ3+ clones among T cell clones derived from intestinal biopsy specimens of coeliac patients.[21] Secondly, we show that changes in numbers and subsets of TcR γδ+ cells are restricted to the gut epithelium and cannot be detected in peripheral blood. Finally, we observed that TcR γδ+ IEL remained high not only in coeliac patients on a gluten free diet but also in seven coeliac patients whose mucosa had returned to normal in spite of a gluten containing diet. Only one of these patients had a low count of TcR γδ+ IEL. In this patient, dermatitis herpetiformis and coeliac disease, defined according to ESPGAN criteria, had subsided. Frozen tissue from the time of active disease was not available, so that we could not determine whether this low count was indeed related to the recovery.

The mechanisms involved in the expansion of TcR γδ+ cells are not elucidated.[9] In mice, expansion of a subset of TcR γδ+ IEL has been linked to one MHC class II haplotype.[22] However, it remains unclear whether this expansion is driven by the MHC antigen itself or by the MHC antigen associated with an exogenous or an endogenous peptide. In coeliac disease, a direct link between the expansion of TcR γδ+ IEL and coeliac disease associated MHC class II haplotypes is suggested by a recent study which shows a significant association between the increased density in TcR γδ+ IEL and the genetic markers of CD, DR3, DQA, and DQB in the healthy first degree relatives of coeliac disease patients.[23] Yet, not all relatives with at risk HLA haplotypes had increased counts of TcR γδ+ cells. It is possible that cytokines released during viral or bacterial infections can enhance the epithelial expression of MHC class II antigens to the level necessary to trigger expansion of TcR γδ+ cells in genetically prone patients. Alternatively, the effect of the MHC class II antigens may be exerted via the presentation of peptides to which TcR γδ+ IEL are reactive. The present study as well as other studies show that changes in TcR γδ+ IEL are observed independently of the diet, indicating that expansion of TcR γδ+ IEL is not driven by gliadin derived peptides. The demonstration that some subsets of TcR γδ+ cells react against mycobacterial or autologous heat shock proteins[9 24] leads us to suggest that the accumulation of heat shock proteins as a result of the gut bacterial colonisation or because of epithelial damage may perhaps contribute to trigger expansion of TcR γδ+ IEL in predisposed patients.

The contribution of TcR γδ+ IEL to the pathogenesis of the mucosal atrophy is also unclear. The persistent increase in TcR γδ+ IEL in coeliac disease patients who have recovered a normal mucosa suggests that TcR γδ+ IEL do not induce directly the epithelial damage. This hypothesis is supported by recent studies which show an increased number of TcR γδ+ IEL in latent coeliac disease several years before appearance of mucosal atrophy.[23 25] Their abnormal expansion in the gut epithelium of coeliac patients may perhaps disturb the local immune mechanisms controlling normal tolerance to dietary antigens. The observation in mice that

Figure 3: Double staining of an intestinal frozen tissue section in a patient from group C who has recovered a nearly normal mucosa after 15 years on a normal diet. The section was first stained by a mixture of anti-TiγA, anti-Vδ2, and δTCS1 mAb and revealed in brown using peroxidase labelled antisera and diaminobenzidine. The same section was then incubated with the pan-anti TcR γδ cells, TcRδ1 mAb, and revealed in blue by alkaline-phosphatase labelled reagents and fast-blue.[19] The brown cells are probably double stained cells (single arrow) whereas the numerous blue cells only stained with TcRδ1 mAb (double arrows). Note that most positive cells are located in the epithelium. The blue staining of the brush border is caused by endogeneous alkaline phophatase.

TcR γδ+ IEL are able to abrogate oral tolerance to a dietary antigen could be relevant to this hypothesis.[26]

In contrast to TcR γδ+ cells, TcR αβ+ cells underwent striking changes depending on the disease activity. These changes mainly affected CD8+ IEL. Variations in the numbers of CD4+ IEL and of TcR αβ+ lamina propria cells were much less significant. Thus, in patients on a gluten containing diet with total villous atrophy and signs of intestinal mononuclear cell activation, CD8+TcR αβ+ IEL were considerably increased. Preliminary results indicate that the latter cells express the heterodimeric form of CD8 (N Cerf-Bensussan, J DiSanto, N Brousse, D Guy-Grand, unpublished data) and may derive, based on recent results in mice, from thymodependent T cells primed in Peyer's patches by intraluminal antigens.[10] In contrast and in agreement with a previous observation,[8] counts of TcR αβ+ cells decreased or returned to normal simultaneously with epithelial recovery and the disappearance of signs of intestinal mononuclear cell activation after gluten free diet. These findings suggest that expansion of CD8+TcR αβ+ IEL is directly driven by gluten and that gluten responsive CD8+TcR αβ+ IEL may be directly involved in the pathogenesis of the villous atrophy. This hypothesis is reinforced by observations made in a small number of coeliac disease patients who have apparently become tolerant to gluten as evidenced by clinical, biological, and histological criteria. In patients with complete epithelial recovery and disappearance of signs of intestinal mononuclear cell activation, the number of TcR αβ+ IEL was indeed comparable with that of age matched controls. However, other studies (for example using T cell clones derived from the intestinal mucosa) will be needed to ascertain the reactivity of TcR αβ+ IEL toward gliadin derived peptides in coeliac disease.

Finally our study raises the question of the enduring character of coeliac disease. According to ESPGAN criteria,[16 17] coeliac disease is considered as a permanent state of intolerance to gluten which requires a life long gluten free diet. Some of our patients, after having undergone gluten challenge, showed a good clinical tolerance to gluten. We allowed these selected patients to eat a normal diet in the long term. Careful follow up had indicated that epithelial recovery could occur with time.[12 13] We show here that epithelial recovery is accompanied by the disappearance of several stigmata of abnormal intestinal immune reactivity. This suggests that in some cases of coeliac disease mechanisms involved in oral tolerance may overcome the continuing abnormal reactivity of intestinal lymphocytes to gliadin. However, the appreciable increase in TcR γδ+ IEL observed in several of these patients recalls that observed in latent coeliac disease[23 25] and can be taken as a strong argument for maintaining the gluten free diet as the standard treatment for coeliac disease, at least until the precise role of TcR γδ+ IEL in the pathogeny of coeliac disease is elucidated.

TABLE V *Numbers of βF1+ lymphocytes in epithelium and lamina propria (LP) according to the degree of villous atrophy (values mean (SD))*

	No of βF1+ IEL/100 EC		*No of βF1+ lymphocytes/field*	
	Surface epithelium	*Crypt epithelium*	*Upper LP*	*Pericryptic LP*
Coeliac patients				
STVA/TVA n=18	43 (10)*	8 (3)§	34 (11)π	19 (9)‡
Moderate VA n=7	26 (10)†	5 (2)‡	26 (8)‡	30 (8)‡
Normal mucosa n=9	21 (5)‡	5 (2)‡	24 (6)‡	23 (9)‡
Paediatric controls n=15	14 (7)	3 (1)	24 (6)	17 (6)
Adult controls n=15	17 (8)	4 (1)	43 (27)**	45 (28)**

VA=villous atrophy; STVA=subtotal villous atrophy; TVA=total villous atrophy.
* $p<0.001$ compared with controls and patients with normal mucosa, $p<0.01$ compared with patients with moderate VA; † $p<0.05$ compared with adult controls, $p<0.01$ compared with paediatric controls, not significant compared to patients with normal mucosa; ‡ not significant compared with controls; § $p<0.01$ compared with adult controls, $p<0.001$ compared with paediatric controls, $p<0.05$ compared with patients with moderate VA or normal mucosa; π $p<0.01$ compared with paediatric controls, $p<0.02$ compared with patients with normal mucosa, not significant compared with adult controls and to patients with moderate VA; ** counts performed in 8 out of the 15 adult controls.

TABLE VI *Variations in the numbers of intraepithelial lymphocytes (IEL)/100 surface epithelial cells according to the degree of villous atrophy (values mean (SD))*

	No of IEL/100 EC revealed by monoclonal antibodies:				
	Anti-CD3	*βF1*	*Anti-CD8*	*Anti-CD4*	*TcRδ1*
Coeliac patients					
STVA/TVA (n=18)	68 (18)*	43 (10)§	49 (11)π	3·6 (·9)‡‡§§	15 (9)ππ
Moderate VA (n=7)	46 (9)†	26 (10)	27 (11)**	1·3 (0·8)	17 (13)
Normal mucosa (n=9)	38 (15)‡	21 (5)	24 (7)††	2·1 (1·9)	14 (10)
Paediatric controls (n=15)	17 (8)	14 (7)	16 (8)	1·3 (0·7)	3·1 (2·4)
Adult controls (n=15)	22 (10)	17 (8)	16 (10)	3·1 (1·5)	3·6 (2·7)

Subtotal villous atrophy; total villous atrophy; villous atrophy.
* $p<0.001$ compared with controls, $p<0.01$ compared with patients with moderate VA or normal mucosa; † $p<0.001$ compared with controls; ‡ $p<0.01$ compared to adult controls, $p<0.001$ compared with paediatric controls; § cf Table V; π $p<0.001$ compared with controls, to patients with moderate or normal mucosa; ** $p<0.05$ compared with controls; †† $p<0.05$ compared with paediatric controls, not significant compared with adult controls ($p>0.05$); ‡‡ Decimals were only indicated for small numbers; §§ $p<0.01$ compared with paediatric controls, not significant compared with adult controls ($p>0.01$); ππ cf Table II.

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