

Effect of diet and age on jejunal and circulating lymphocyte subsets in children with coeliac disease: persistence of CD4⁺CD8⁻ intraepithelial T cells through treatment

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Abstract

Monoclonal antibodies were used to determine the relative numbers of T lymphocyte subsets in 61 jejunal biopsies and in peripheral blood of 35 children with coeliac disease, and of 13 healthy controls. The T cell numbers in the lamina propria were unaffected by a change from gluten-free to gluten containing diet in the patients. The number of intraepithelial lymphocytes (where the CD8⁺ cells predominated) were significantly raised in patients taking gluten. Ten to 20% of the patients' intraepithelial CD3⁺ (mature T) cells expressed neither CD8 nor CD4 surface antigens. This CD4⁺CD8⁻ T cell population persisted through gluten elimination and challenge. The circulating lymphocyte subsets showed little variation with the diet although there was a marked increase in the proportion (14.9%) of CD4⁺CD8⁻ T cells in patients during gluten elimination. In the histologically normal jejunal mucosa from control subjects, the age of the subject showed a positive correlation with villus intraepithelial CD3⁺ and CD8⁺ cells, and crypt intraepithelial CD4⁺ cells. No clear cut effect of age was observed on lamina propria lymphocyte counts of the controls, or on the lymphocyte counts in jejunal mucosa of the coeliac patients. The observed CD3⁺CD4⁺CD8⁻ lymphocytes may represent activated cells unable to present their surface antigens, or they may be $\gamma\delta$ -receptor bearing T cells, which could have a significant role in the pathogenesis of coeliac disease.

The human jejunal mucosa harbours a large population of lymphocytes. In lamina propria the lymphocytes are predominantly of the CD4⁺ phenotype, with about 30% of CD8⁺ cells also present, whereas approximately 85% of the intraepithelial lymphocytes belong to the CD8⁺ subset.^{1,2}

While it is well known that the relative size of body lymphoid tissues is great in infancy and diminishes later in childhood, virtually nothing has been reported on the effect of age on intestinal lymphoid cell subsets.

In patients with active coeliac disease the counts of lamina propria lymphocytes and the relative numbers of their subsets are comparable to those in healthy controls.^{3,4} The numbers of intraepithelial lymphocytes are increased,⁵ the majority of them being of the CD8⁺ subpopulation.^{3,4,6,7} A subgroup of T intraepithelial lymphocytes in adults with active coeliac disease was recently observed, expressing neither CD4⁺

nor CD8⁺ surface markers. The authors suggested that these T cells could be in altered functional state, possibly as committed cytotoxic lymphocytes, and hence not able to fully express their distinct phenotypic markers.⁸

In peripheral blood the proportions of the different lymphocyte subpopulations show little variation with dietary changes in coeliac patients.^{1,9}

We studied the lymphocyte subpopulations in jejunal mucosa and peripheral blood of children with coeliac disease through the diagnostic dietary regimen, and also in healthy control children, in order to clarify the developmental changes in different T cell subsets in intestinal mucosa, and their role in coeliac disease.

Methods

PATIENTS

During the diagnostic evaluation¹⁰ of 35 children (19 boys) with coeliac disease, a part of the jejunal biopsy specimen was set aside for this study. The pretreatment biopsy (n=11) was taken at the mean age of 7.8 years (range: 0.6-15.5). The second biopsy, taken after an average of 4.3 years on gluten free diet, was available for 20 patients. Of 17 patients who had been taking gluten for a period averaging 4.6 months, we could study the third, or postchallenge biopsy. In 11 children both the second and the third biopsies were available for this study. All children showed jejunal villous atrophy in the first biopsy and marked clinical improvement on gluten free diet. Six patients have not yet been challenged with gluten; they were all four years or older at presentation, two of them have dermatitis herpetiformis and additional two diabetes mellitus.

CONTROLS

The control group consisted of 13 children (six boys) with mean age of 8.2 years (range: 0.65 to 15.2) who were studied for growth retardation. They had neither symptoms nor findings of gastrointestinal disease. The jejunal mucosa was normal in each of them.

TISSUE PROCESSING

Intestinal biopsy specimens were taken from the proximal jejunum with the paediatric Crosby capsule. The specimen was divided immediately into two parts. One half was processed for routine histological examination. The other half

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was embedded in OCT compound (Tissue-Tek 4538, Miles Laboratories Inc, Naperville, Illinois), frozen in freon 22 suspended over liquid nitrogen, and stored at -70°C . For further processing 5 μ sections were cut, air dried for one hour, and fixed in acetone at $+4^{\circ}\text{C}$ for 10 minutes. After a 30 minute incubation in chloroform the sections were washed in Tris buffer (0.01 M Tris 7-9 in 0.14 M NaCl, pH=7.4, Sigma, St Louis, Missouri).

MONOCLONAL ANTIBODIES

A panel of commercially available mouse monoclonal antibodies was used to detect lymphocyte surface markers in the specimens. Anti-CD3 antibodies included T1 (Dakopatts, Copenhagen, Denmark), Leu 4 (Beckton Dickinson, Mountain View, California) and OKT3 (Ortho Diagnostic Systems, Raritan, New Jersey); anti CD4 antibodies used were T4 (Coulter Immunology, Hialeah, Florida), Leu 3a 3b (Beckton Dickinson) and OKT 4 (Ortho); CD8 antigens were detected by OKT8 monoclonal antibody (Ortho).

IMMUNOHISTOCHEMICAL METHOD

The fixed frozen sections were incubated with the monoclonal antibody and washed with Tris buffer. Peroxidase conjugated rabbit antimouse immunoglobulin (Dakopatts, Copenhagen, Denmark) in 1:10 dilution was added. After another wash an incubation with peroxidase conjugated goat antirabbit IgG serum (Tago, Burlingame, California) followed, and a final wash was performed. All of the incubations were carried out at room temperature for 30 minutes; the duration of each wash was 10 minutes. The peroxidase stain was developed with hydrogen peroxide activated AEC (3-amino-9-ethylcarbazole, Sigma, St Louis, Missouri) for 20 minutes, and lightly counterstained with hematoxylin. The specimens were mounted in Gurr Aquamount (BDH Chemicals Inc, Poole, England). Similarly processed sections without the first monoclonal antibody were used as controls. They showed only minimal traces of staining indicating that endogenous peroxidase activity had been removed in the process.

EVALUATION OF THE MICROSCOPIC SPECIMENS

The microscopic evaluation was done by one of the authors (AA), who was blinded to the diagnosis. The cell density in the lamina propria was determined with a 0.045 \times 0.045 mm graticule fitted to the microscope eyepiece counting the number of cells in at least 30 graticules, and expressing the result as cells/mm².

In immunoperoxidase stained sections the epithelial cells were only faintly visible, making intraepithelial lymphocyte counting in relation to epithelial cells unreliable. Therefore, we compared this method to counting the intraepithelial lymphocytes along the above-mentioned graticule in H&E stained sections, and found an excellent correlation (Table I). Thus, in further

TABLE I *Intraepithelial lymphocyte numbers in H&E stained sections of jejunal mucosa of children with coeliac disease, and in controls (mean (SEM))*

	Children with coeliac disease			Controls n=13
	Before treatment n=11	Gluten free diet n=20	Gluten challenge n=17	
IEL/mm	63 (10)†	42 (3)*	83 (5)‡	30 (3)
IEL/100 epith cells	33 (7)†	18 (2)*	42 (4)‡	12 (2)

*Difference from the controls; p<0.05; †p<0.01; ‡p<0.001.

work, intraepithelial lymphocytes were counted per epithelial length and expressed as cells/mm of epithelium.

PERIPHERAL BLOOD LYMPHOCYTE COUNTING

While taking routine blood tests at the time of the intestinal biopsy a blood sample for lymphocyte subset determination was drawn. The numbers of CD3 (OKT3), CD4 (OKT4) and CD8 (OKT8) positive cells were counted using a previously described indirect immunofluorescent technique.¹¹

STATISTICAL ANALYSIS

For calculation of the statistical significance of the observed differences between the patients and the controls, or patients at different stages of dietary treatment, a computer program was used, applying Student's *t* test, the Pearson χ^2 test, Fisher's exact test or regression analysis, whichever was applicable.¹²

Results

IMMUNOHISTOCHEMICAL STAINING

The peroxidase labelled cells were readily identifiable on microscopy. The different monoclonal antibodies used for CD3⁺ and CD4⁺ cells gave similar results for their respective subpopulations. Of the CD3⁺ cell antibodies Leu4 produced staining that was easiest to read in the conditions described. Therefore it was used for the subsequent comparisons. Similarly, T4 was used as the reference antibody for CD4⁺ cells.

LAMINA PROPRIA LYMPHOCYTES

In the lamina propria of coeliac children all T cell subset counts were lower during gluten elimination and challenge than in the biopsies before

TABLE II *Number of lymphocytes in the lamina propria of the jejunal biopsies of children with coeliac disease and controls stained with the different antibodies (mean (SEM/mm²))*

Type of cell	Children with coeliac disease			Controls n=13
	Before treatment n=11	Gluten free diet n=20	Gluten challenge n=17	
CD3	1707 (136)	1565 (82)*	1468 (65)*	1963 (189)
CD4	1323 (135)	1123 (50)*	1076 (78)*	1502 (159)
CD8	756 (117)	680 (36)*	655 (35)*	919 (104)
CD4 /CD8 ratio	1.93	1.70	1.67	1.70

*Difference from the controls; p<0.05.

treatment, or in the controls (Table II). In the 11 patients in whom jejunal biopsies during gluten free diet and gluten challenge were available, the dietary switch caused no observable changes in any lamina propria cell counts (data not shown).

The CD4⁺/CD8⁺ ratio in the patients remained similar to that of the controls and virtually unchanged throughout the follow up.

The lamina propria CD3⁺ and CD4⁺ lymphocyte counts showed a weak positive correlation ($R=0.43$, $p=0.14$, and $R=0.41$, $p=0.17$, respectively) with the age in the controls. In the patients no correlation with age could be observed.

INTRAEPIHELIAL LYMPHOCYTES

Intraepithelial T cell numbers in the surface epithelium showed significant changes in concert with the dietary changes (Table III). During gluten containing diet the numbers of all lymphocyte subpopulations were significantly higher than in patients on gluten elimination diet, or in the controls.

The numbers of CD3⁺ intraepithelial lymphocytes equalled the sum of CD4⁺ and CD8⁺ cells in the controls (Fig 1). In the coeliac patients, however, the numbers of CD3⁺ cells exceeded the sum of CD4⁺ and CD8⁺ cells. The percentage of CD3⁺ cells unidentified by either CD4 or CD8 antibodies was 10.5% before treatment, 14.9% during gluten-free diet and 20.6% during gluten challenge. The 'unidentified' cell population was significantly greater ($p<0.05$) in patients than in the controls (0.1%).

In the crypt epithelium the T cell numbers were roughly one-third of those within surface epithelium, and the changes during dietary changes closely resembled those observed among surface intraepithelial lymphocytes (Fig 2).

The age of the subject had an observable positive correlation with surface CD3⁺ ($R=0.65$, $p<0.02$) and CD8⁺ ($R=0.54$, $p=0.056$), intraepithelial lymphocyte counts in the structurally normal biopsies from the controls. The correlation coefficients of other lymphocyte subpopulations with age were smaller, and statistically insignificant, but invariably positive. In the coeliac patients, whether treated or not, the correlation between age and intraepithelial lymphocyte numbers was obscured.

PERIPHERAL BLOOD LYMPHOCYTES

In peripheral blood the numbers of CD4⁺ and CD8⁺ T cells showed little or no variation with

TABLE III Number of lymphocytes (cells/mm of epithelium) within surface epithelium of the jejunal biopsies of children with coeliac disease and controls stained with the different antibodies (mean (SEM/mm²))

Type of cell	Children with coeliac disease			Controls n=13
	Before treatment n=11	Gluten free diet n=20	Gluten challenge n=17	
CD3	60.9 (10.2)†	39.0 (3.3)*	85.0 (6.3)‡	27.9 (3.6)
CD4	3.9 (0.8)*	2.3 (0.3)	3.1 (0.4)*	1.5 (0.4)
CD8	50.6 (8.1)*	30.9 (3.0)	64.4 (4.4)‡	26.3 (4.0)
CD4 /CD8 ⁺ ratio	0.077	0.081	0.049	0.066

*Difference from the controls; $p<0.05$; † $p<0.01$; ‡ $p<0.001$.

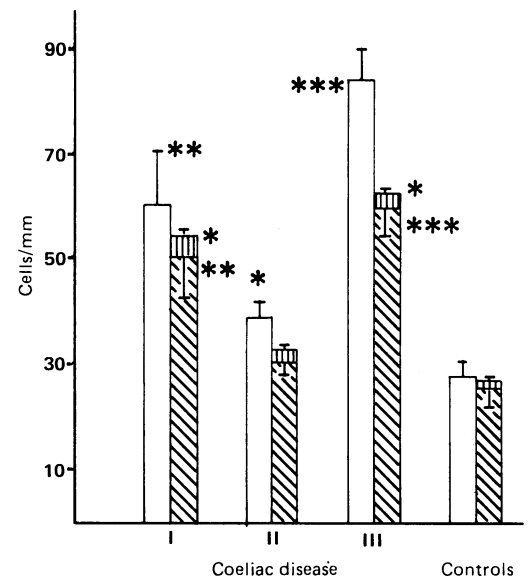


Figure 1: Lymphocyte subpopulations within the jejunal surface epithelium of children with coeliac disease at different stages of dietary treatment and controls. Symbols: Open columns=CD3⁺ cells; hatched columns=CD4⁺ cells; vertically hatched columns=CD8⁺ cells. I=patients before treatment; II=patients on gluten-free diet; III=patients after gluten challenge. *, **, *** denote a significant difference from the controls, with p values less than 0.05, 0.01 and 0.001, respectively.

regard to the dietary changes in the coeliac patients. The CD4⁺/CD8⁺ ratio showed neither a correlation with, nor a constant difference from that in the intestine. The percentage of CD3⁺ cells unidentified by either CD4 or CD8 antibodies was relatively high in the treated patients (14.9%), and lower in patients before treatment (0.0%) or during gluten challenge (4.9%), or in the controls (3.5%).

Discussion

The results of an immunohistochemical study of lymphocyte subsets in children with coeliac disease and control subjects are presented. Six of the patients do not yet fulfill the ESPGAN diagnostic criteria of coeliac disease,¹⁰ but they have other diseases associated with it, and were well past infancy at the initial jejunal biopsy showing typical histological lesions. Of more than 100 children attending our gastrointestinal services, who have had severe villous atrophy at two years of age or later, every one has eventually fulfilled the ESPGAN criteria of coeliac disease.

In the lamina propria CD4⁺ was the predomi-

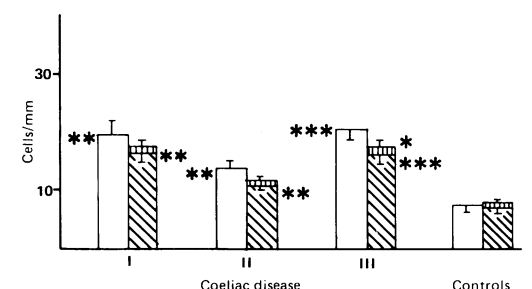


Figure 2: Lymphocyte subpopulations within the jejunal crypt epithelium of controls and children with coeliac disease at different stages of dietary treatment. Symbols: see legend to Figure 1.

nating T cell type. The observed low LP cell counts in patients during later diagnostic stages, and as compared with the controls (Table II), is an unexpected finding, the significance of which is further obscured by the fact that no decrease was observed in the 11 patients in whom both the pre and postchallenge biopsies were available.

In lamina propria the sum of CD4⁺ and CD8⁺ cells exceeded the number of CD3⁺ cells in all the subject groups studied. This difference remained virtually unchanged throughout the diagnostic stages, and resembled the findings in healthy controls. This observation, being in contrast with that made of intraepithelial lymphocytes, indicates a marked difference in surface marker expression and differentiation between lamina propria and intraepithelial lymphocyte T cells, which may be because of weak expression of CD3 antigens, or, hypothetically to the presence of double positive (CD4⁺8⁺) cells. The relative constancy of these findings in the patients throughout the study, and similarity to the findings in controls, suggest that in coeliac disease the main T cell abnormality could be found in the intraepithelial lymphocyte, and not in lamina propria lymphocyte populations.

In contrast with the relatively small changes observed in lamina propria, the intraepithelial T cell numbers showed marked variations in concert with the dietary changes in the coeliac patients, as has been reported.^{3,4,6} We observed a similar gap between the numbers of CD3⁺ cells and the sum of CD4⁺ and CD8⁺ cells as Jenkins and coworkers⁸ in their adult patients with active disease. We also found a significant proportion of CD3⁺ cells unidentified by either CD4 or CD8 antibodies in the histologically normal jejunal biopsies of treated coeliac patients, who also showed somewhat higher intraepithelial lymphocyte counts than the controls. This finding could have at least two explanations. First, there may be minor errors in the most strictly observed diet, causing intraepithelial lymphocyte activation and inconsistent surface marker expression.¹³ An alternative explanation is that the 'unidentified' CD3⁺ cells could represent the recently described CD3⁺4⁻8⁻, $\gamma\delta$ -receptor bearing cells.^{14,15} Such a cell population, being present even in the structurally normal jejunal mucosa of coeliac patients, could have an important role in the pathogenesis of the disease. The appearance of a similar T cell population in the peripheral blood of the patients when gluten is withheld, could reflect a decrease in the local immunological and chemotactic activity, which conceivably would release the CD3⁺4⁻8⁻ cells into the circulation. We are presently pursuing to define the T cell receptors of this cell population.

James *et al*¹⁶ reported that among isolated intestinal lamina propria lymphocytes the proportion of CD4⁺ cells was greater than in peripheral blood. We could not confirm this observation. The reason may be in different methods of intestinal cell enumeration, or in the fact that we used only proximal jejunal biopsies, whereas

James *et al* isolated cells from various parts of the GI canal. We, like Rodgers *et al*¹⁷ found similar CD4⁺/CD8⁺ ratios in lamina propria and peripheral blood of healthy control subjects.

The observed positive correlations between age and CD3⁺ and CD8⁺ intraepithelial lymphocyte counts in our small number of healthy controls warrant further study. If confirmed, this observation of a trend contrasting the general tendency seen in – for example, peripheral blood and lymphatic tissues, might provide material for better understanding of mucosal immune responses. In the patients with coeliac disease the rather weak correlations observed may be obscured by the inevitable patient-to-patient variation in the strictness of the gluten-free diet, or possibly by age-related changes in the peculiar CD3⁺4⁻8⁻ intraepithelial lymphocyte population.

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