

Serum granzymes and CD30 are increased in children's milk protein sensitive enteropathy and celiac disease

Merja T. Augustin, MD,^a Jorma Kokkonen, MD,^c Riitta Karttunen, MD,^b
and Tuomo J. Karttunen, MD^a Oulu, Finland

Background: Cow's milk protein sensitive enteropathy (CMSE) and celiac disease (CD) associate with upregulation of local intestinal immune responses, including activation of cytotoxic lymphocytes.

Objective: We investigated whether this upregulation can be detected at the systemic level by analyzing serum concentrations of granzymes A (GrA) and B (GrB), soluble Fas, and CD30.

Methods: Fifty-seven children with a final diagnosis of CMSE in 23 subjects (18 untreated and 5 on a diet), 20 untreated CD patients, and 14 control subjects with no gastrointestinal disease were examined by endoscopy for gastrointestinal complaints. Duodenal biopsies were studied for basic histology, CD3, $\alpha\beta$ and $\gamma\delta$ T-cell receptors (TCRs), and serum samples by commercial ELISA assays.

Results: Concentrations of GrA and GrB were significantly higher in untreated CMSE and in CD as compared with the control subjects. Measurable GrB was present in only 20% of the control subjects but in 100% of patients with CMSE. CD30 was similarly increased in both untreated CMSE and CD cases, whereas in treated CMSE cases the concentrations were equal to the control subjects. All groups showed similar soluble Fas. The numbers of duodenal CD3⁺, $\alpha\beta$ - and $\gamma\delta$ -TCRs correlated with the serum granzyme and CD30 levels.

Conclusion: The intestinal immune response in CMSE and CD is manifested by the increase in serum GrA, GrB, and CD30; the measurement of these markers provides a new practical and objective complementary means for diagnosis and assessment of treatment response. However, this has to be confirmed by more studies before routine use. (*J Allergy Clin Immunol* 2005;115:157-62.)

Key words: Children, food allergy, enteropathy, coeliac disease, serum, granzyme, CD30, Fas, cytotoxic lymphocytes, duodenum

Abbreviations used

CD:	Celiac disease
CMSE:	Cow's milk protein sensitive enteropathy
GrA:	Granzyme A
GrB:	Granzyme B
IEL:	Intraepithelial lymphocytes
LNH:	Lymphoid nodular hyperplasia
NK cell:	Natural killer cell
sFas:	Soluble Fas
TCR:	T-cell receptor
TIA-1:	T-cell restricted intracellular antigen type 1

Immune-mediated sensitivity to food antigens is an increasingly common problem affecting up to 7% of children. During infancy, the common type is food allergy mediated by immediate hypersensitivity.¹ In older children delayed responses activated locally on the gastrointestinal mucosa are increasingly involved; cow's milk protein sensitive enteropathy (CMSE) is the most familiar clinical entity. The prevalence of CMSE has been estimated to be about 2% to 3% in unselected subjects at school age.²⁻⁴ Food-borne symptoms have also been associated with irritable bowel syndrome.⁵ In all these states diagnostic modalities have so far remained incomplete.

Symptoms of delayed food hypersensitivity vary and are often inconclusive. They frequently include recurrent or chronic abdominal pains and diarrhea or constipation, bloating, blood in the stool, and anemia.^{6,7} Many of these symptoms, shared by several gastrointestinal or other diseases, are commonly encountered in clinical practice. The definite diagnosis of food hypersensitivities is difficult; skin tests and measurement of allergen-specific IgE antibodies usually remain negative in CMSE.^{1,8} Accordingly, conclusive diagnosis of food hypersensitivity may require both exclusion of conditions with similar symptoms and a tedious elimination challenge test, in which both positive and negative results are sometimes difficult to interpret.⁹

We and others have recently shown immunohistochemically that CMSE in children is associated with an increment in the duodenal intraepithelial lymphocytes (IELs) including T-cell restricted intracellular antigen type

From ^athe Departments of Pathology and ^bMedical Microbiology, Oulu University, and ^cthe Department of Children and Adolescents, Oulu University Hospital.

Supported by the Research Foundation of Danisco Finland and the Research Foundation of the Finnish-Norwegian Medical Foundation.

Received for publication April 8, 2004; revised October 7, 2004; accepted for publication October 7, 2004.

Available online November 19, 2004.

Reprint requests: Tuomo J. Karttunen, MD, Department of Pathology, University of Oulu, POB 5000, FIN-90014 University of Oulu, Finland.
E-mail: tuomo.karttunen@oulu.fi.

0091-6749/\$30.00

© 2005 American Academy of Allergy, Asthma and Immunology

doi:10.1016/j.jaci.2004.10.009

1 (TIA-1),^{10,11} perforin, and granzyme A (GrA) (Augustin et al, unpublished observations) positive and $\gamma\delta$ T-cell receptor (TCR) bearing T lymphocytes.^{2,12} These findings indicate that abnormal lymphocyte response involving lymphocyte cytotoxicity may be important in the pathogenesis of this type of food hypersensitivity. In some patients, the mucosal abnormality may be widespread in the gastrointestinal tract, extending to the ileal and colonic mucosa.⁷ A similar but much more abundant increase in cytotoxic IELs in intestinal mucosa is characteristic of celiac disease (CD),¹³⁻¹⁵ in which the expression of apoptosis-inducing FasL is also increased in intestinal lymphocytes¹⁵; these abnormalities lead to an enterocyte apoptosis and villous atrophy.¹⁶

There is an urgent need for noninvasive tests that would help in the categorization of chronic gastrointestinal symptoms into those with and without a role in immunologically mediated response to food constituents. In addition to preliminary diagnosis of CMSE, such tests might possibly be used to complement serologic tests for CD such as transglutaminase antibody determination to increase their specificity by helping to correctly classify borderline results and thus avoid unnecessary endoscopies.^{17,18} Furthermore, such tests could be used in objective monitoring of the effect of dietary elimination and challenge and so help in confirming the diagnosis and in determining appropriate dietary restrictions.

Hoping to find new clinically usable diagnostic tools for CMSE and CD in the present preliminary study, we have tested whether mucosal immune activation can be estimated by analyzing the serum levels of different types of immune activation marker. Serum determination of two well-defined and characterized markers of cytotoxic activation, GrA and granzyme B (GrB), was used to determine cytotoxic activation.¹⁹ The overall lymphocyte activation was monitored by serum CD30 determination, which is a member of the tumor necrosis factor/nerve growth receptor superfamily and is mainly released by CTLs and natural killer cells (NKs) dependent on their activation.²⁰ Serum sFas, an apoptosis-related protein,²¹ was investigated because an increase in the apoptosis rate has been observed in CMSE (Augustin et al, unpublished observations) and in CD.¹⁶ Finally, because significant differences in some of these markers were apparent between the patient groups, we wanted to see whether there was any relationship between the serum levels and the local immunologic activation in the intestinal mucosa as shown by the density of different categories of duodenal IELs.

METHODS

Patients

This study consisted of an unselected series of 57 children with a mean age of 10.2 years (range, 4-15 years; 22 males) referred to the Oulu University Hospital, Finland, for pediatric gastroenterologic consultation because of refractory gastrointestinal complaints.

Twenty-three of the patients (mean age, 9.2 years; range, 4-15 years; 11 males) were diagnosed as CMSE cases. They had been

referred for various symptoms; the dominant symptom was abdominal pain in 11 patients, diarrhea or loose mucous stools in 12, four of whom had blood in stools. Diagnosis of CMSE was based on an open elimination and challenge test that resulted in the disappearance of the symptoms on a milk elimination diet (2 weeks) and their reappearance in an open challenge test with low lactose milk powder and written symptom diaries. The test continued for another 2 weeks if symptoms did not manifest earlier.^{2,6} Abdominal pain, diarrhea or loose mucous stools, and exacerbation of dermatitis were each considered as a positive result. In cases with atopic symptoms, possible IgE-mediated food allergy was assessed by determination of milk-specific IgE concentration (RAST test); all 8 remained negative. Of the patients with CMSE, 18 (mean age, 9.3 years; range, 4-15 years; 9 males) were studied before the definite diagnosis was assessed, whereas 5 (mean age, 9 years; range, 6-14 years; 2 males) were studied during the elimination diet.

The other group consisted of 20 patients with CD (mean age, 10.5 years; range, 5-15 years; 5 males) diagnosed according to the acknowledged criteria.²² Only those with subtotal or total villous atrophy with crypt hyperplasia in the duodenal sample were included. Referral symptoms were diarrhea or loose mucous stools in 12 patients, abdominal pain in 5, growth retardation in 2, and recurrent vomiting in 1 patient. Four of the CD patients showed patchy villous atrophy in the upper duodenal samples and were considered to have mild CD.

The control group consisted of 14 subjects (mean age, 11.4 years; range, 6-15 years; 6 males) referred and examined for exclusion of any definite gastrointestinal diseases. Four subjects with both recurrent abdominal pains and diarrhea and 2 subjects with abdominal pains were tested with a similar milk elimination and challenge test as the CMSE group, but the results remained negative. The occasional abdominal pains of the other 5 control subjects indicated no need for elimination and challenge test, and the symptoms subsided during a long follow-up of at least 6 months with milk-containing diet. None of the 3 subjects with eating disorders had any abdominal symptoms, which would have indicated the need to perform elimination and challenge tests. After an adequate examination and follow-up it was concluded that none of the 14 control subjects had a manifest gastrointestinal disease and/or that the symptoms had remitted.

The Ethics Committee for Clinical Science of Oulu University Hospital approved the study protocol. Informed, written consent was obtained from all the parents of the study children.

Procedures

Serum samples were evaluated by the ELISA method for GrA and GrB (Pelikine Human Granzyme A and Pelikine Human Granzyme B; CLB, Amsterdam, The Netherlands), serum soluble Fas (R&D Quantikine human sFas; Minneapolis, Minn), and CD30 (Bender MedSystems GmbH, Vienna, Austria). All samples were analyzed simultaneously by using the manufacturer's instructions. GrA and GrB concentrations were expressed as units/mL and those of soluble Fas (sFas) and CD30 as pg/mL.

The biopsy samples from the bulb of the duodenum were embedded in an OCT compound (Miles Laboratories, Elkhart, Ind), frozen in liquid nitrogen, and stored at -70°C until analysis. Sections of 5 μm were fixed in methanol for 3 minutes at $+4^{\circ}\text{C}$ and stained by using the Vectastain Elite ABC kit (Vectastain PK 6102; Vector Laboratories, Burlingame, Calif) and antibodies for CD3 (mAb Leu-4; Becton Dickinson, San Jose, Calif), $\alpha\beta$ -TCR molecules (MAB βF1 ; Endogen Inc, Woburn, Mass) and $\gamma\delta$ -TCR molecules (Mab TCR- γ ; Endogen Inc), as described previously.¹² The cell densities were counted by a person blinded for all other data.

TABLE I. Numbers of duodenal intraepithelial CD3⁺ T cells and $\alpha\beta$ - and $\gamma\delta$ -TCR-bearing cells (cells/mm) in control children and in children with untreated or treated CMSE or untreated CD

Cell type	CTRL (n = 14)	CMSE (untreated) (n = 14)	CMSE (treated) (n = 3)	CD (n = 20)	
CD3	14 (16), 6-28	26 (30), 14-67	14 (16), 13-23	54 (58), 33-100	
αβ-TCR	12 (13), 4-33	20 (23), 9-50	10 (15), 10-26	35 (35), 9-63	
γδ-TCR	1 (1), 0-5	4 (6), 0-22	2 (2), 1-3	21 (23), 4-53	
CD3	αβ-TCR		γδ-TCR		
CTRL/CMSE (untreated)	<i>P</i> = .005	CTRL/CMSE (untreated)	<i>P</i> = .003	CTRL/CMSE (untreated)	<i>P</i> = .015
CTRL/CD	<i>P</i> < .001	CTRL/CD	<i>P</i> < .001	CTRL/CD	<i>P</i> < .001
CMSE (untreated)/CD	<i>P</i> < .001	CMSE (untreated)/CD	<i>P</i> = .008	CMSE (untreated)/CD	<i>P</i> < .001
CMSE (treated)/CD	<i>P</i> = .006	CMSE (treated)/CD	<i>P</i> = .035	CMSE (treated)/CD	<i>P</i> = .001

Median (mean) and range are indicated. Groups were compared with the Kruskal-Wallis test; significant differences are indicated. CTRL, Control subjects.

Statistical methods

The data were analyzed by SPSS (Release 11.5; SPSS Inc, Chicago, Ill). Because of skewed, non-normal distribution of serum concentrations and cell densities in most of the patient groups, the nonparametric Mann-Whitney *U* test and Kruskal-Wallis test were used to estimate the significance of differences between the groups. Results are expressed as medians, and the mean is also indicated. A *P* value of $<.05$ was considered significant.

RESULTS

Endoscopic and immunohistochemical findings

Lymphoid nodular hyperplasia (LNH) of the duodenal bulb was a characteristic endoscopic finding in 8 (35%) of 23 CMSE subjects, but it was also found in 2 CD patients and 2 control subjects (data not shown). None of the CMSE patients or control subjects had the villous atrophy or crypt hyperplasia typically seen in all CD cases. Endoscopic esophagitis was found in 3 CMSE patients and 2 control subjects and endoscopic gastritis in 5 CMSE, 3 CD, and 4 control patients. All IEL counts were higher in CD patients than among the other groups (Table I). The untreated CMSE patients had significantly higher densities of CD3, $\alpha\beta$ - and $\gamma\delta$ -positive T cells in duodenal samples than the control subjects. However, in all CMSE cases on a diet, the densities were comparable with the control subjects (Table I).

Serum concentrations of GrA, GrB, sFas, and CD30

The serum concentrations of GrA, GrB, and CD30 in children with CMSE, CD, and control subjects are shown in Figs 1 through 3. Subjects with untreated CMSE showed significantly increased concentrations as compared with control subjects and the treated CMSE group. In CD, these levels were similarly increased as compared with control subjects, but they remained somewhat lower than in the CMSE patients (Figs 1 through 3). Only 20% of the children in the control group (3/15) and in the treated CMSE group (1/5) showed measurable levels of GrB, whereas 100% (18/18) in the untreated CMSE and 65% (13/20) in the CD group showed a measurable amount of

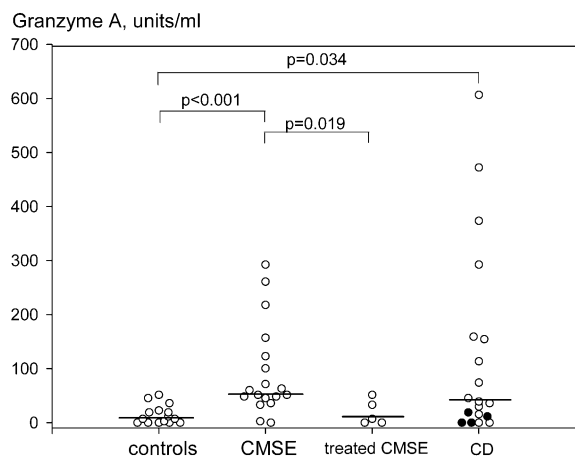


FIG 1. Scatterplot showing serum granzyme A concentration (units/mL) in control subjects, patients with CMSE, treated CMSE, and CD. In the CD group subjects with mild disease (see Methods) are indicated by black dots. Lines indicate median values. Statistical significance was assessed with the Kruskal-Wallis test.

GrB ($P < .001$; χ^2 test). By using the reference values provided by the kit manufacturer (below 60 units/mL for GrA and below 40 units/mL for GrB), both control subjects and patients with treated CMSE showed normal concentrations. With CD, GrA was increased in 40% of cases (8/20; $P = .011$ compared with control subjects) and GrB in 30% (6/20; $P = .032$ compared with control subjects). In CMSE 50% of patients (9/18) showed an increase in GrA ($P = .002$ compared with control subjects) and 72% (13/18) in GrB ($P < .001$ compared with control subjects). Four patients with CD showing patchy villous atrophy had significantly less GrA (median, 6 units/mL; range, 0-19 units/mL) than CD patients with continuous villous atrophy (median, 60 units/mL; range, 0-607 units/mL; $P = .029$). All the IEL subtype densities in these 4 patients were, however, at the same level as the remaining CD patients. Serum sFas concentrations were similar in the control subjects (median, 4200 pg/mL, range, 3100-5200 pg/mL), in untreated CMSE (median, 3950 pg/mL; range, 2600-5400 pg/mL), treated CMSE

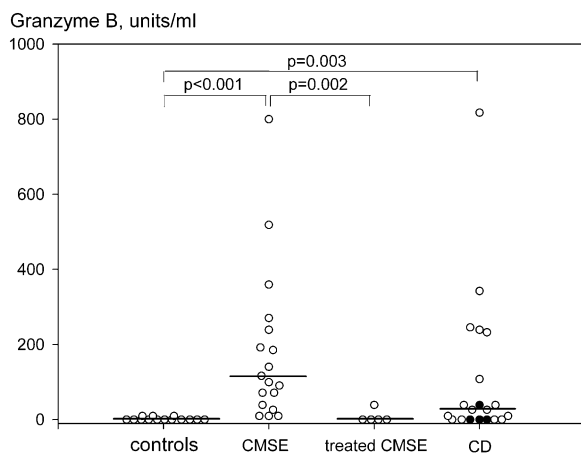


FIG 2. Scatterplot showing serum granzyme B concentration (units/mL) in control subjects, patients with CMSE, treated CMSE, and CD. In the CD group subjects with mild disease (see Methods) are indicated by *black dots*. Lines indicate median values. Statistical significance was assessed with the Kruskal-Wallis test.

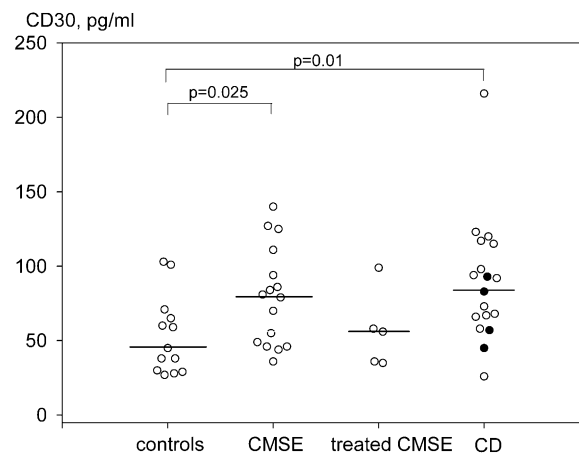


FIG 3. Scatterplot showing serum CD30 concentration (pg/mL) in control subjects, patients with CMSE, treated CMSE, and CD. In the CD group subjects with mild disease (see Methods) are indicated by *black dots*. Lines indicate median values. Statistical significance was assessed with the Kruskal-Wallis test.

(median, 4000 pg/mL; range, 3200–4700 pg/mL), and CD (median, 4200 pg/mL; range, 3200–5200 pg/mL).

There was a high positive correlation between GrA and GrB concentrations ($r = 0.76$; $P < .001$, Spearman) and between the granzymes and CD30 ($r = 0.47$; $P < .001$) in the whole study group. In subgroup analysis, the markers showed similar interdependence in CD ($P < .026$). In CMSE, only GrA and GrB showed a significant correlation ($P < .001$), and in the control group none of the markers showed any significant correlation.

Intraepithelial lymphocyte densities and serum markers

We then analyzed the correlations of IEL densities and serum marker concentrations to find possible evidence for interdependence. Serum GrA across the whole study material ($N = 57$) showed a significant correlation with the numbers of all cell categories including CD3, $\alpha\beta$ - and $\gamma\delta$ -TCR-bearing IELs (Table II), whereas GrB correlated with the numbers of CD3 and $\gamma\delta$ -TCR-bearing IELs (Table II). CD30 concentration showed significant correlation with all cell types; correlation with the numbers of $\gamma\delta$ -TCR-bearing IELs was highly significant. In contrast, sFas showed no significant relationship with cell counts across the whole material, but in subgroup analysis, sFas and CD3 counts correlated in CD ($r = 0.513$; $P = .021$). In untreated CMSE, the number of $\gamma\delta$ -TCR-bearing IELs correlated with CD30 concentration ($r = 0.707$; $P = .01$) and in the control group with GrB ($r = 0.719$; $P = .004$).

DISCUSSION

The major finding in this study was that for the first time the immune response taking place locally on the mucosa

of the gastrointestinal tract could be evaluated by using a measurement of serum samples. Serum GrA, GrB, and CD30 levels were significantly increased in children with untreated CMSE, whereas the concentrations were low among control subjects without evidence of gastrointestinal disease. Untreated CD patients showed an increment quite similar to the CMSE group. The finding that the serum concentrations of these proteins were at normal levels in patients with treated CMSE with an appropriate elimination diet indicates that the serum response is dependent on antigen exposure and is not a constant phenomenon in patients with CMSE.

Significant correlations between serum GrA, GrB, and CD30 levels and the numbers of CD3⁺ duodenal IELs including $\gamma\delta$ -TCR-bearing T cells strongly suggest that serum concentrations are indeed related to immunologic activation in the intestine. Our results indicate that the measurement of serum granzymes and CD30 might provide a novel noninvasive way of monitoring immune-mediated intestinal diseases both during the initial diagnosis and in the assessment of treatment response. So far, there have been no such tests for these purposes, although measures of this kind would often be useful in the confirmation of diagnosis of CMSE and other food-allergic disorders^{1,9} and possibly even in the assessment of the clinical significance of results of serologic tests for CD.^{17,18}

The endoscopic and immunohistochemical findings of duodenal biopsies confirm our previous findings concerning the mucosal state in the duodenum of patients with CMSE. We found LNH in one third of the CMSE subjects and a significant elevation in the densities of intraepithelial CD3, $\alpha\beta$ - and $\gamma\delta$ -positive T cells in the duodenum,^{12,23} indicating that CMSE should be included in the differential diagnostic consideration when increase in IELs, a hallmark of CD,¹⁸ is seen in biopsies, or LNH is seen at endoscopy.

In CMSE, the concentrations of GrA and GrB showed most discriminative values in comparison with control subjects. Detectable levels of GrB were present in about 20% of control subjects; all concentrations were within the normal range, but 100% of subjects with CMSE showed measurable GrB, and in 72% the value exceeded the normal range. Similarly high prevalence figures were seen in CD as well but, interestingly, not to the same extent as in subjects with CMSE. Although the detection level is likely to depend on the sensitivity of the assay and to be a matter of variation between laboratories, and the normal range may show differences in different populations, these findings point out the magnitude of the difference between control subjects and patients with intestinal immune activation. Granzymes are a family of several granule-associated proteases; types A and B are the most abundant. They are released from the granules of CTLs and NK cells that induce apoptosis of the target cells by several, so far largely unknown mechanisms.²⁴ The immunologic synapse forms a tight seal that directs the cytolytic granzymes to the target cell and keeps them from leaking out, but elevated serum levels are seen in conditions of intense cell-mediated activation such as rheumatoid arthritis.¹⁹ Accordingly, increased serum levels of granzymes have been detected in viral,¹⁹ parasitic,²⁵ and bacterial infections²⁶ and in autoimmune diseases such as scleroderma²⁷ and rheumatoid arthritis,^{19,28} in which synovial granzyme concentration is similarly increased. However, it is apparent that the increment in serum granzyme concentrations as such has no specific relation to the etiologic factor inducing lymphocyte activation.

Serum CD30 was significantly increased in both CMSE and CD, but there was much overlap with the control subjects, indicating a less discriminative value compared with the granzyme assays. CD30 is mainly released from activated lymphocytes and NK cells.²⁰ It may identify T_H2-type clones, but its *in vivo* relevance is still a matter of debate, because high serum levels of soluble CD30 have been found in both T_H1- and T_H2-dominated disorders.²⁹ Increased levels have been detected in respiratory allergy but not in IgE-mediated food allergy.³⁰ On the other hand, in infants with cow's milk allergy, cow's milk protein-specific T cells expressed much higher levels of CD30 by flow cytometry than the same cells of infants diagnosed as having tolerance.³¹ These discrepant findings about CD30 and food protein reactivity may be related to the heterogeneity of the disease in terms of IgE dependence and the T_H1/T_H2 paradigm.

The sFas concentration was similar in all subjects and showed no discriminating value or correlation with the other serum markers. Only in CD was there a correlation with serum sFas and the number of duodenal CD3⁺ T cells. Fas (APO-1 or CD95) is a cell surface glycoprotein expressed on rapidly proliferating cells and is strongly upregulated on activated lymphocytes. It has been shown that serum levels of sFas during the attack phase in patients with allergic rhinitis are significantly lower than those in healthy individuals. In contrast, serum sFas levels in patients with bronchial asthma are increased during the

TABLE II. Correlation matrix of serum GrA, GrB, sFas, and CD30 concentrations and the counts of duodenal intraepithelial CD3⁺ T cells and $\alpha\beta$ - and $\gamma\delta$ -TCR-bearing cells in the whole study material (N = 51; CD30, N = 46)

	CD3	$\alpha\beta$ -TCR	$\gamma\delta$ -TCR
GrA	0.285 *	0.278 *	0.285 *
GrB	0.286 *	0.245	0.314 *
sFas	0.238	0.239	0.251
CD30	0.417**	0.373 *	0.497 ***

Correlation coefficient (Spearman's rho).

**P* < .05.

***P* < .01.

****P* < .001.

attack phase.³² These findings, together with our observations, indicate that serum sFas concentration is an unpredictable marker in allergic or other hypersensitive diseases, but it is a potential indicator of immunologic activation in CD.

The current screening tools for CD, such as the determination of tissue transglutaminase antibodies, have relatively high specificity and sensitivity in the range of 95% when human tissue transglutaminase is used as an antigen.^{17,18} However, there are several pitfalls in the serologic tests for CD, related to methodology, autoantibody class, amount of ingested gluten, and associated conditions such as diabetes and liver diseases; these factors cause both false positive and negative results.¹⁸ Although the levels of GrA and GrB were significantly increased in CD, the values exceeding the normal range were present in only 40% and 30% of cases, respectively. GrA or GrB determination has probably no use as such in the diagnosis of CD. However, granzyme levels showed association with the extent of villous atrophy; association with GrA was statistically significant. These findings suggest that granzyme determinations might have some value in CD screening as a complementary test; increased levels suggest a possibly clinically significant mucosal immune activation and thus help to determine the need for additional or follow-up investigations in cases with borderline or inconsistent results in the standard CD screening tests. Further studies are also needed to see whether a demonstration of significant decrease in these immune activation markers could be applied in assessing treatment response in CD.

An interesting and unexpected finding was that the GrB level in CMSE subjects was higher than in CD subjects, even though the level is supposed to have a direct correlation with the density of IELs and cytotoxic activity in the small intestinal mucosa. It is higher in CD subjects¹⁴ and far lower in CMSE patients.¹¹ In CMSE the distribution of mucosal changes varies, and in many cases, in addition to the abnormalities in the proximal small intestine, ileal and colon mucosa may also show an increase in IELs.²³ We thus speculate that, like the extent of villous changes in CD associating with serum GrA levels, the serum concentration of GrB in CMSE may depend on the extent of mucosal lesions; higher values possibly associate

with a more extensive intestinal immune response. However, more studies are necessary to confirm this idea.

In conclusion, our preliminary report suggests that upregulated immune response of the gastrointestinal tract in CMSE and CD can indeed be assessed by determining serum granzyme and CD30 concentrations. This finding introduces a new and complementary means for the diagnostic assessment of these diseases as well as for the scientific study of the skewing of gut-associated lymphoid tissue in hypersensitive disorders and autoimmune states. For the objective diagnosis of food hypersensitivity we look forward to establishing an elimination–challenge test based on the decline in serum markers of the cytotoxic immune response during the elimination period and their rise during the challenge. However, we still need more comprehensive studies to see the detailed association between these markers and the skewing of the mucosal lymphoid system.

Acknowledgment: The immunohistochemical analyses of CD3, $\alpha\beta$ - and $\gamma\delta$ -TCR were performed at the Coeliac Service Laboratory, University of Tampere, Tampere, Finland.

REFERENCES

1. Sicherer SH. Food allergy. *Lancet* 2002;360:701-10.
2. Kokkonen J, Haapalahti M, Laurila K, Karttunen TJ, Maki M. Cow's milk protein-sensitive enteropathy at school age. *J Pediatr* 2001;139:797-803.
3. Kokkonen J, Tikkanen S, Savilahti E. Residual intestinal disease after milk allergy in infancy. *J Pediatr Gastroenterol Nutr* 2001;32:156-61.
4. Kokkonen J, Haapalahti M, Tikkanen S, Karttunen R, Savilahti E. Gastrointestinal complaints and diagnosis in children: a population based study. *Acta Paediatr* 2004;93:880-6.
5. Talley NJ, Spiller R. Irritable bowel syndrome: a little understood organic bowel disease? *Lancet* 2002;360:555-64.
6. Kokkonen J, Ruuska T, Karttunen TJ, Niinimäki A. Mucosal pathology of the foregut associated with food allergy and recurrent abdominal pains in children. *Acta Paediatr* 2001;90:16-21.
7. Wakefield AJ, Murch SH, Anthony A, Linnell J, Casson DM, Malik M, et al. Ileal-lymphoid-nodular hyperplasia, non-specific colitis, and pervasive developmental disorder in children. *Lancet* 1998;351:637-41.
8. Sampson HA. Food allergy. *J Allergy Clin Immunol* 2003;111(suppl):S540-7.
9. Bock SA. Diagnostic evaluation. *Pediatrics* 2003;111:1638-44.
10. Hankard GF, Matarazzo P, Duong JP, Mougenot JF, Navarro J, Cezard JP, et al. Increased TIA1-expressing intraepithelial lymphocytes in cow's milk protein intolerance. *J Pediatr Gastroenterol Nutr* 1997;25:79-83.
11. Augustin M, Karttunen TJ, Kokkonen J. TIA1 and mast cell tryptase in food allergy of children: increase of intraepithelial lymphocytes expressing TIA1 associates with allergy. *J Pediatr Gastroenterol Nutr* 2001;32:11-8.
12. Kokkonen J, Holm K, Karttunen TJ, Maki M. Children with untreated food allergy express a relative increment in the density of duodenal $\gamma\delta$ T cells. *Scand J Gastroenterol* 2000;35:1137-42.
13. Oberhuber G, Vogelsang H, Stolte M, Muthenthaler S, Kummer AJ, Radaszkiewicz T. Evidence that intestinal intraepithelial lymphocytes are activated cytotoxic T cells in celiac disease but not in giardiasis. *Am J Pathol* 1996;148:1351-7.
14. Ciccocioppo R, Di Sabatino A, Parroni R, D'Alo S, Pistoia MA, Doglioni C, et al. Cytolytic mechanisms of intraepithelial lymphocytes in coeliac disease (CoD). *Clin Exp Immunol* 2000;120:235-40.
15. Di Sabatino A, Ciccocioppo R, D'Alo S, Parroni R, Millimaggi D, Cifone MG, et al. Intraepithelial and lamina propria lymphocytes show distinct patterns of apoptosis whereas both populations are active in Fas based cytotoxicity in coeliac disease. *Gut* 2001;49:380-6.
16. Moss SF, Attia L, Scholes JV, Walters JR, Holt PR. Increased small intestinal apoptosis in coeliac disease. *Gut* 1996;39:811-7.
17. Maki M, Mustalahti K, Kokkonen J, Kulmala P, Haapalahti M, Karttunen T, et al. Prevalence of celiac disease among children in Finland. *N Engl J Med* 2003;348:2517-24.
18. Green PH, Jabri B. Coeliac disease. *Lancet* 2003;362:383-91.
19. Spaeny-Dekking EH, Hanna WL, Wolbink AM, Wever PC, Kummer AJ, Swaak AJ, et al. Extracellular granzymes A and B in humans: detection of native species during CTL responses in vitro and in vivo. *J Immunol* 1998;160:3610-6.
20. Del Prete G, De Carli M, Almerigogna F, Daniel CK, D'Elios MM, Zancuoghi G, et al. Preferential expression of CD30 by human CD4+ T cells producing Th2-type cytokines. *FASEB J* 1995;9:81-6.
21. Ho CY, Wong CK, Ko FW, Chan CH, Ho AS, Hui DS, et al. Apoptosis and B-cell lymphoma-2 of peripheral blood T lymphocytes and soluble fas in patients with allergic asthma. *Chest* 2002;122:1751-8.
22. Walker-Smith JA, Guandalini S, Schmitz J, Schermerling DH, Visakorpi JK. Revised criteria for diagnosis of celiac disease. *Arch Dis Child* 1990;65:909-11.
23. Kokkonen J, Karttunen TJ, Niinimäki A. Lymphonodular hyperplasia as a sign of food allergy in children. *J Pediatr Gastroenterol Nutr* 1999;29:57-62.
24. Lieberman J. The ABCs of granule-mediated cytotoxicity: new weapons in the arsenal. *Nat Rev Immunol* 2003;3:361-70.
25. Hermens CC, Konijnenberg Y, Mulder L, Loc C, van Deuren M, van der Meer JW, et al. Circulating concentrations of soluble granzyme A and B increase during natural and experimental *Plasmodium falciparum* infections. *Clin Exp Immunol* 2003;132:467-72.
26. Lauw FN, Simpson AJ, Hack CE, Prins JM, Wolbink AM, van Deventer SJ, et al. Soluble granzymes are released during human endotoxemia and in patients with severe infection due to gram-negative bacteria. *J Infect Dis* 2000;182:206-13.
27. Kahaleh MB, Fan PS. Mechanism of serum-mediated endothelial injury in scleroderma: identification of a granular enzyme in scleroderma skin and sera. *Clin Immunol Immunopathol* 1997;83:32-40.
28. Tak PP, Spaeny-Dekking L, Kraan MC, Breedveld FC, Froelich CJ, Hack CE. The levels of soluble granzyme A and B are elevated in plasma and synovial fluid of patients with rheumatoid arthritis (RA). *Clin Exp Immunol* 1999;116:366-70.
29. Horie R, Watanabe T. CD30: expression and function in health and disease. *Semin Immunol* 1998;10:457-70.
30. Blanco Quiros A, Arranz E, Hoyos C, Carrasco JA, Andion R. Different serum interleukin-12 and sCD30 levels in food- and pollen-sensitized children. *Pediatr Allergy Immunol* 1999;10:235-40.
31. Schade RP, Van Ieperen-Van Dijk AG, Versluis C, Van Reijssen FC, Kimpen JL, Bruijnzeel-Koomen CA, et al. Cell-surface expression of CD25, CD26, and CD30 by allergen-specific T cells is intrinsically different in cow's milk allergy. *J Allergy Clin Immunol* 2002;109:357-62.
32. Kato M, Nozaki Y, Yoshimoto T, Tamada Y, Kageyama M, Yamashita T, et al. Different serum soluble Fas levels in patients with allergic rhinitis and bronchial asthma. *Allergy* 1999;54:1299-302.