

Deposition of eosinophil-granule major basic protein and expression of intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 in the mucosa of the small intestine in infants with cow's milk-sensitive enteropathy

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Background: Cow's milk-sensitive enteropathy (CMSE) is an important cause of chronic diarrhea and failure to thrive in infancy. The immunopathology of the mucosal lesion associated with CMSE has not yet been described.

Objectives: This study investigated the eosinophil activation and the role of adhesion molecules in the pathogenesis of intestinal mucosal damage associated with CMSE.

Methods: Twenty-one patients with chronic diarrhea and abnormal mucosa on duodenal biopsy specimens were included. The patients had negative responses to skin prick tests and RASTs with milk. Fourteen patients were diagnosed with CMSE by milk challenge test and were designated as the CMSE group. Seven patients with no milk intolerance were defined as the non-CMSE group. Four infants with frequent vomiting and no mucosal abnormalities were also studied as the control group. Immunohistochemical stains for eosinophil major basic protein (MBP), vascular cell adhesion molecule-1 (VCAM-1), and intercellular adhesion molecule-1 on endoscopic duodenal biopsy specimens were performed.

Results: The degree of eosinophil degranulation, as evidenced by localization of extracellular MBP, was significantly greater in the CMSE group compared with the non-CMSE and control groups ($P < .05$). Expression of VCAM-1 on mononuclear cells was higher in the CMSE group compared with the non-CMSE and control groups ($P < .05$). The severity of villous atrophy was positively correlated with the deposition of MBP ($r = 0.79$, $P < .001$).

Conclusion: These results strongly suggest eosinophils and VCAM-1 are implicated in the pathogenesis of mucosal damage associated with CMSE. (*J Allergy Clin Immunol* 1999;103:1195-201.)

Key words: Cow's milk-sensitive enteropathy, chronic diarrhea, major basic protein, vascular cell adhesion molecule-1, intercellular adhesion molecule-1

Cow's milk-sensitive enteropathy (CMSE) is a temporary disorder in infancy characterized by abnormal small intestinal mucosa during the period in which cow's milk is included in the diet. The abnormality is reversed by a cow's milk-free diet but recurs with a subsequent resumption of cow's milk.¹ The onset of symptom may be acute but is more often insidious,² resulting in chronic and persistent diarrhea. Walker-Smith et al³ and Manuel et al⁴ previously described small intestinal biopsy specimens from patients with milk intolerance as containing patch lesions with partial villous atrophy. However, it is not always possible to differentiate CMSE from other causes of chronic diarrhea with the same gross and histologic appearances.^{1,4}

The exact mechanism causing the loss of surface epithelial cells leading to villous atrophy in CMSE is at present not clear. The role of IgE-mediated hypersensitivity in the acute cutaneous reactions of patients with cow's milk allergy is well established.^{5,6} However, the immune responses associated with delayed-onset gastrointestinal symptoms remain undetermined.⁷⁻⁹

In this study we measured eosinophil granule major basic protein (MBP), vascular cell adhesion molecule-1 (VCAM-1), and intercellular adhesion molecule-1 (ICAM-1) directly on duodenal biopsy specimens to investigate their roles in the pathogenesis of mucosal damage associated with CMSE.

METHODS

Patients and control subjects

Twenty-one patients with chronic diarrhea who underwent both milk challenge tests and duodenal biopsies were included. All infants had been fed with cow's milk formula since birth. They all had negative responses to skin prick tests and RASTs with milk. The subjects were divided into 2 groups (ie, the CMSE and non-CMSE groups) by the responses to the elimination and reintroduction of cow's milk.

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TABLE I. Diagnostic challenge tests with cow's milk

Day 1	¼- to ½-strength formula; 10 mL at 9 AM; 10 mL at 10 AM and NPO for observation
Day 2	Full-strength formula; 10 mL, 20 mL, 30 mL, 40 mL, and 60 mL at 1-hour intervals and NPO for observation
Day 3	Normal milk intake appropriate for age and observation

NPO, Nothing by mouth.

TABLE II. Clinical features after cow's milk challenge in patients with CMSE

Reaction	Onset	Colic	Vomiting	Diarrhea	Abdominal distension
Intermediate (n = 9)	1-24 h	1/9	5/9	9/9	5/9
Late (n = 5)	>24 h	0/5	1/5	5/5	1/5

Abbreviations used

CMSE: Cow's milk-sensitive enteropathy
ICAM-1: Intercellular adhesion molecule 1
MBP: Major basic protein
VCAM-1: Vascular cell adhesion molecule 1

The CMSE group consisted of 14 patients, aged from 24 days to 8 months, all demonstrating signs of failure to thrive. These patients became asymptomatic soon after the elimination of milk from the diet. Diagnostic milk challenges (Table I) were performed with parental consent, causing a recurrence of symptoms including vomiting, diarrhea, and abdominal distension. The time of symptom onset after challenge was between 1 and 24 hours in 9 patients and after 24 hours in 5 patients (Table II). These patients belonged to the intermediate- and late-reacting groups of patients with cow's milk allergy as described by Firer et al.⁸ Patients with bacterial and protozoal diseases were excluded.

The non-CMSE group consisted of 7 patients, aged 18 days to 11 months. Diarrhea persisted after elimination of milk from the diet (parenteral nutrition or protein hydrolysate). Six patients in this group tolerated a soy formula followed by reintroduction of milk to the diet after an initial clinical improvement. One patient died of necrotizing enterocolitis. Rotavirus was identified in 3 patients, and *Escherichia coli* was identified in 1 patient.

Four infants, aged 2 to 8 months, in whom a duodenal endoscopy was performed for frequent vomiting, served as control subjects after their biopsy specimens were diagnosed as normal.

Duodenal biopsy

Duodenal biopsy specimens were obtained during upper gastrointestinal endoscopy by using a 7.9-mm Olympus GIF XP 20. Sedation with valium (0.3 mg/kg) or meperidine (1 to 2 mg/kg) was required in some infants. Each infant had a biopsy as soon as possible after admission. Second biopsies were performed in 7 patients from the CMSE group after clinical improvement. The mean interval between the first and second biopsies was 3 weeks.

Immunohistochemical study

Biopsy specimens were immediately fixed in 10% neutral-buffered formalin fixative and embedded in paraffin. They were coded and assessed in blinded fashion by 2 observers. One tissue section (approximately 4 µm) was stained with hematoxylin and

TABLE III. Scoring of MBP staining

Score	Intensity	Extent (%)
0	None	0
1	Weak	<10
2	Moderate	10-25
3	Strong	25-50
4	Very strong	>50

eosin and viewed for villous atrophy and infiltration of lymphoplasmic cells, neutrophils, and eosinophils.

For immunohistochemical staining, sections were placed onto organosilane-coated slides (Fisher Biotech) and stained with a microprobe system (Fisher Scientific Co). After deparaffinization, the slides were pretreated with Autoblocker (Research Genetics) to block endogenous peroxidase followed by proteinase-K (20 µg/mL, Sigma) and 3 washes with PBS. Slides were incubated with specific primary antibodies (1:50) for 20 minutes at 40°C. The mAbs used were anti-ICAM-1 (1H4, NeoMarkers), anti-VCAM-1 (1.4C3, Serotec), and anti-eosinophil MBP (BMK 13, Monosan). An irrelevant mouse IgG1 mAb was used as a negative control. A biotinylated secondary antibody (Histostain-plus kit, Zymed Laboratories Inc) was added and visualized by using avidin-linked peroxidase. The sections were then counterstained with Harris-hematoxylin, mounted in glycerol, and viewed under light microscopy.

Quantification

Extracellular MBP deposition was assessed semiquantitatively on a scale of 0 to 4 in increments of 0.5 according to the extent and intensity of staining¹⁰ (Table III). The endothelial staining of ICAM-1 and VCAM-1 was graded on a scale of 0 to 4 in increments of 0.5 as described by Fukuda et al¹¹: 0, no staining; 1, focal and weak staining; 2, weak staining of most vessels; 3, moderate staining of most vessels; and 4, strong staining of all vessels. The expression of VCAM-1 and ICAM-1 on mononuclear cells was assessed as the number of stained cells per 100 lamina propria cells at a magnification of 400x.

The severity of villous atrophy was assessed on a scale of 0 to 3 (ie, 0, none; 1, mild; 2, moderate; and 3, severe) by the criteria of Whitehead,¹² and the degree of infiltration of cells was assessed on a scale of 0 to 3 (ie, 0, none; 1, mild; 2, moderate; and 3, severe) as described by the Sydney system.¹³

Statistical analysis

Differences between groups were analyzed by using the Mann-Whitney *U* test. Correlation coefficients were calculated with the Spearman rank method. Probability (*P*) values of less than .05 were accepted as statistically significant.

RESULTS

Duodenal biopsy specimens demonstrated patch lesions in both the CMSE and non-CMSE groups. The severity of villous atrophy was not significantly different between the 2 groups.

The extracellular deposition of MBP was mainly present in the lamina propria (Fig 1) and was significantly greater in the CMSE group than in the non-CMSE group and control subjects (median value: 3 vs 1.5 and 3 vs 1, respectively; *P* < .05; Fig 2). The degree of eosinophil infiltration in the CMSE group was not significantly greater than that found in the non-CMSE group and the control group (median value, 1.5 vs 1.5 vs 1, respective-

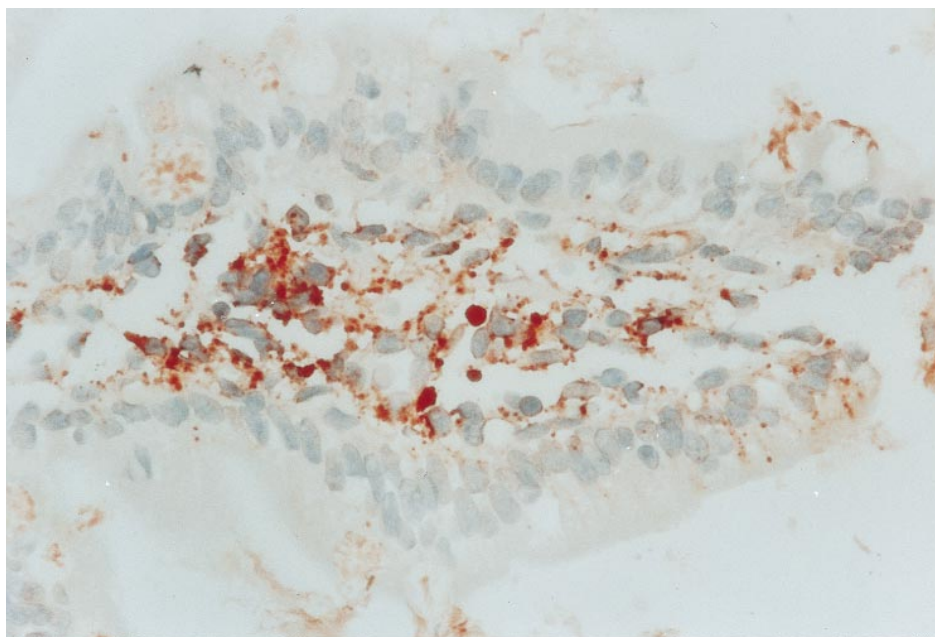


FIG 1. Extracellular deposition of MBP was mainly seen within mucosal lamina propria.

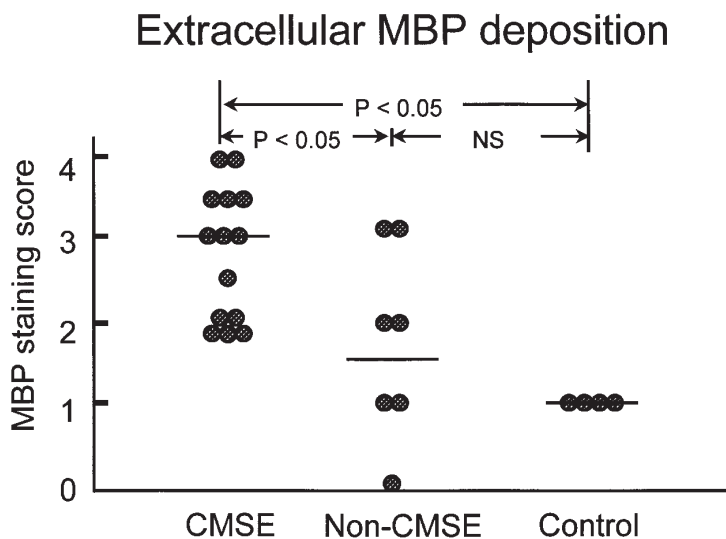


FIG 2. Extracellular deposition of MBP was significantly greater in the CMSE group compared with the non-CMSE and control groups ($P < .05$, respectively). Median values are represented by *horizontal bars*.

cells in both the CMSE and non-CMSE groups. Immunoreactivity for ICAM-1 was also detected in the extracellular space, which may have resulted from the shedding of ICAM-1 (Fig 5). No significant difference was observed between the two groups (Fig 6).

The severity of villous atrophy was positively correlated with the extracellular deposition of MBP ($r = 0.79$, $P < .001$; Fig 7) but not with the expression of adhesion molecules or infiltration of specific inflammatory cells.

Significant recovery of villous atrophy (median value, 2.5 vs 0.5; $P < .001$) and decrease of extracellular MBP deposition (median value, 3.5 vs 2; $P < .05$) were

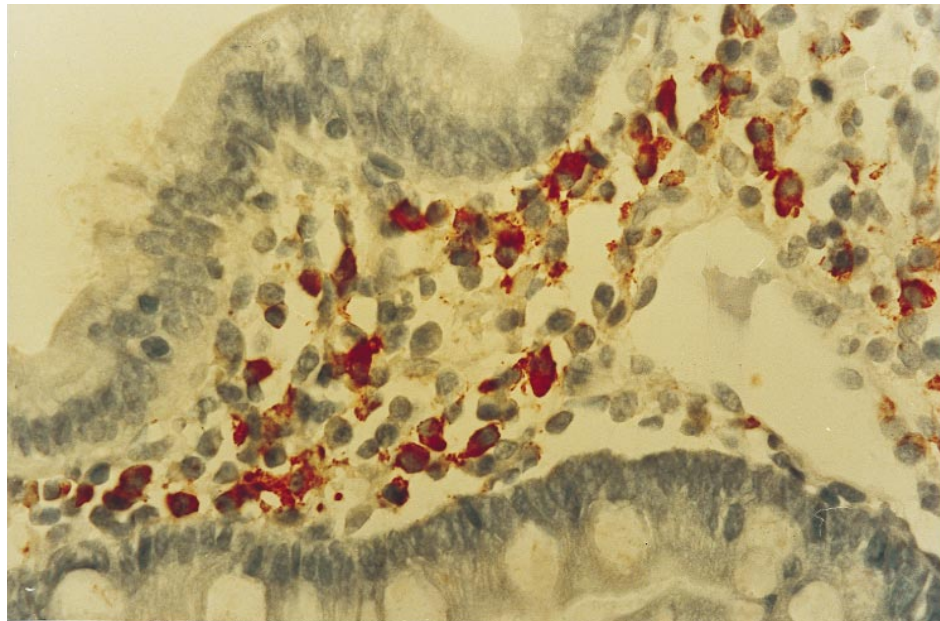


FIG 3. VCAM-1 expression on mononuclear cells was observed within mucosal lamina propria.

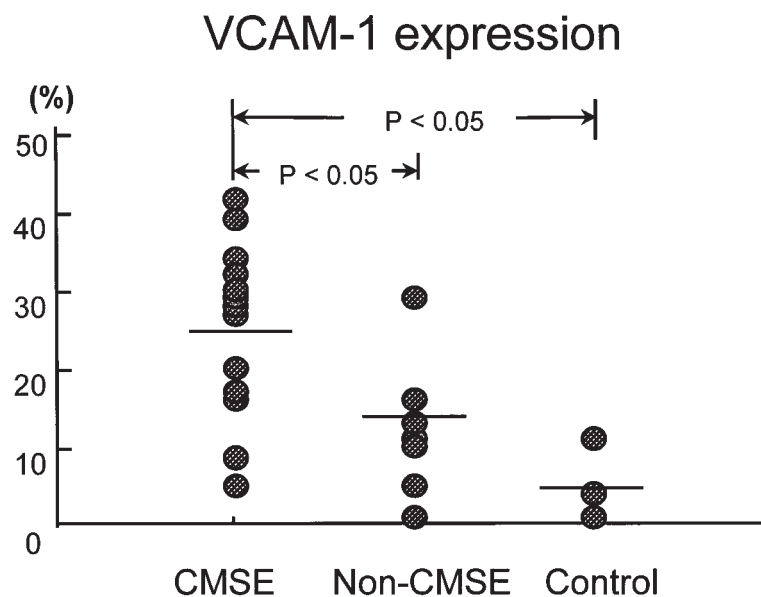


FIG 4. VCAM-1 expression on mononuclear cells in mucosal lamina propria was significantly higher in the CMSE group compared with the non-CMSE and control groups ($P < .05$, respectively). Mean values are represented by horizontal bars.

observed on second biopsies compared with the finding of the first biopsies in 7 patients from the CMSE group. However, there were no significant changes in the expression of VCAM-1 and ICAM-1.

DISCUSSION

In vitro studies have shown that MBP damages tracheal epithelium, intestine, spleen, skin, and PBMCs in a dose-related fashion.^{10,14-16} Our study demonstrated sig-

nificantly increased extracellular MBP deposition in the duodenal mucosa of patients with CMSE. The severity of mucosal villous atrophy was significantly correlated with the extracellular deposition of MBP.

The mucosal infiltration of eosinophils, however, was not significantly greater than that found in the non-CMSE patients and control subjects. The extensive extracellular deposition of MBP in the absence of a prominent accumulation of intact eosinophils in tissue has been

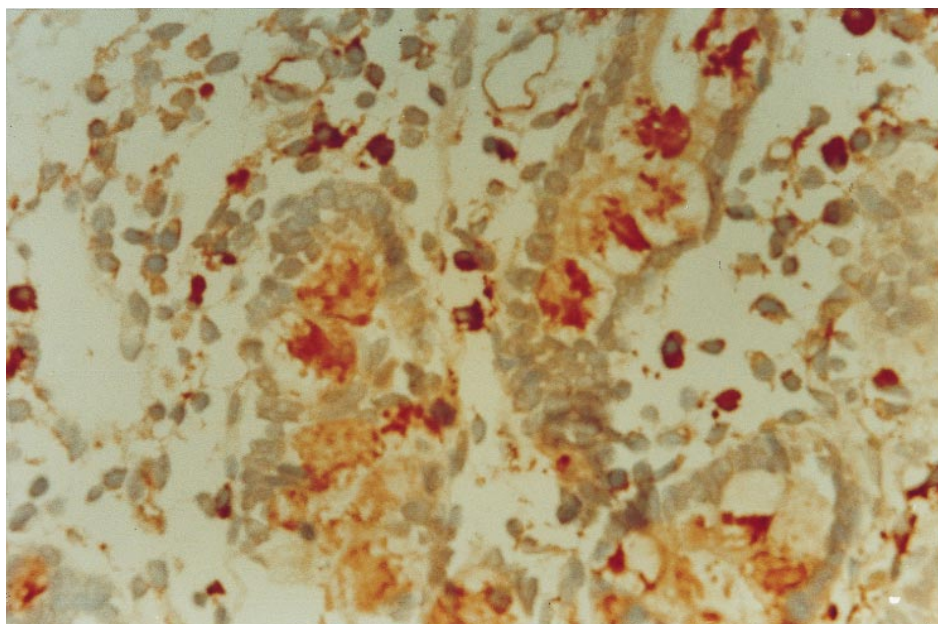


FIG 5. Robust expression of ICAM-1 on vascular endothelial cells and inflammatory cells in mucosal lamina propria.

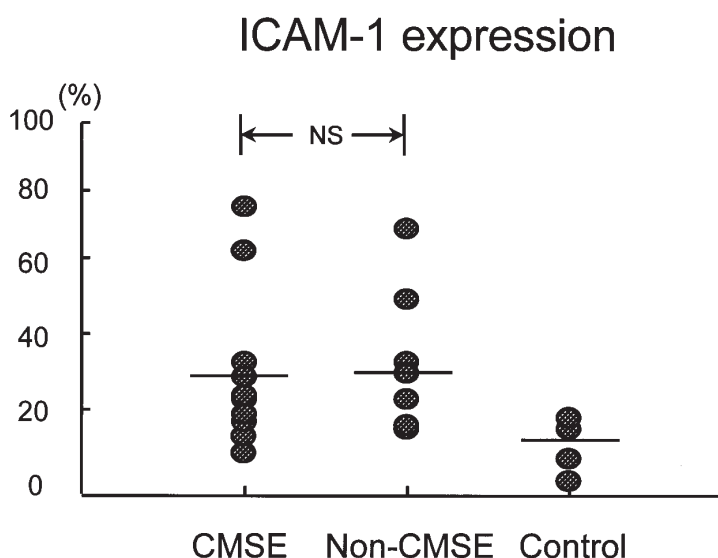


FIG 6. ICAM-1 expression increased in both CMSE and non-CMSE groups without significant intergroup difference. Mean values are represented by horizontal bars. NS, Not significant

observed in previous studies.¹⁸⁻²⁰ Our results suggest that the degranulation of eosinophils, as demonstrated by the localization of MBP, is implicated in the pathogenesis of mucosal damage in CMSE, and an assessment of eosinophil involvement in disease cannot be based simply on numbers of eosinophils in tissue.

We measured the expression of VCAM-1 and ICAM-1 in duodenal mucosa to investigate their roles in CMSE. VCAM-1 expression on endothelial cells was weak or absent and did not show a significant intergroup differ-

ence. However, the expression of VCAM-1 on mononuclear cells was significantly higher in the CMSE group compared with the non-CMSE and control groups. A previous study examining celiac disease showed moderate VCAM-1 expression in the rectal mucosa that was confined largely to mononuclear cells in the lamina propria.²¹ In that study a gluten challenge induced a significant increase in VCAM-1 expression on mononuclear cells with the concurrent rise of CD3⁺ cells. This finding is consistent with our results and suggests that VCAM-

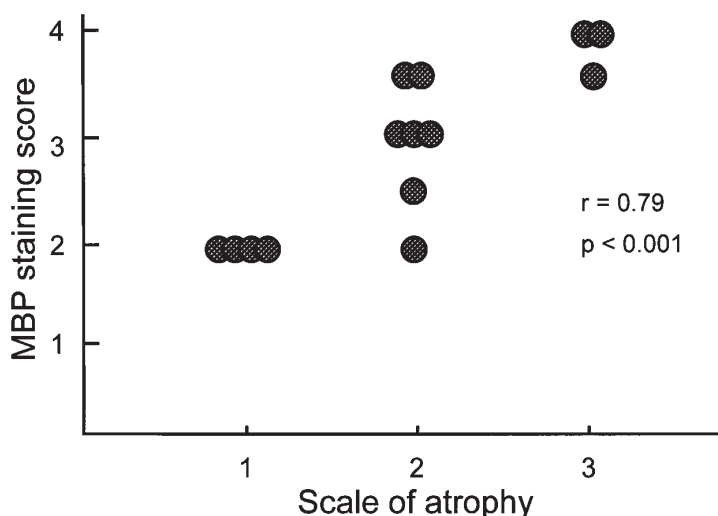


FIG 7. There was a statistically significant relationship between the severity of villous atrophy and the extra-cellular MBP deposition in the CMSE group ($r = 0.79$, $P < .001$).

1-mediated stimulation is required for activation and adhesion of T lymphocytes. A T cell-mediated immune reaction was reported to play a role in the pathogenesis of mucosal damage induced by food-sensitive enteropathy.^{1,17} These findings differed from other studies in inflammatory bowel diseases, such as ulcerative colitis and Crohn's disease, in which VCAM-1 expression appeared to be confined to lymphoid aggregates in both involved and uninvolved segments of colonic mucosa.^{22,23} This suggests that the mechanism of mucosal damage in CMSE is different from that of inflammatory bowel diseases.

Robust expression of ICAM-1 was observed on endothelial cells and inflammatory cells in both the CMSE and non-CMSE groups. A similar increase of ICAM-1 expression has been found in the mucosa of the colon and small intestine in inflammatory bowel diseases.^{22,24}

The significant resolution of villous atrophy and decrease of MBP deposition were observed on second biopsies. However, there was no significant change in the expression of VCAM-1 and ICAM-1. Our results are consistent with the study of Ohkawara et al²⁵ who reported a marked upregulation of ICAM-1 and VCAM-1 in the bronchial mucosa of asthmatic subjects who clinically recovered but still had decreased peak expiratory flow rates (less than 80% of normal control subjects). This indicates that a persistent inflammatory state of the involved duodenal mucosa exists even after the resolution of symptoms.

In conclusion, our results suggest that eosinophils and adhesion molecules are associated with the immunopathologic mechanism of mucosal damage in CMSE. The presence of increased eosinophil degranulation and VCAM-1 expression in duodenal mucosa may be helpful in the characterization and differentiation of food-sensitive enteropathy from other causes of villous atrophy.

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