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Food intolerance and mucosal inflammation

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Abstract

Most infants are immunologically active to develop a tolerance to oligoclonal antigens by producing IgA and introducing regulatory T cells in early infancy. Cytokines and their signalling molecules are important mediators in the intestine regulating both oral tolerance and mucosal inflammation. This system works efficiently in most individuals, but for an as yet undefined reason, some people react to food and other proteins as though they were pathogens, and induce chronic inflammation in the mucosa.

The adverse reaction caused by ingested foods is defined as food intolerance. The clinical features of food intolerance include vomiting, diarrhea, bloody stool, eczema, failure to thrive, and a protean range of other symptoms. Intolerance can be divided into two categories depending on whether or not they are immunologically mediated. Food intolerance and mucosal inflammation are deeply related since tolerance cannot be introduced when there is an inflammation in the intestinal mucosa. Mast cells, eosinophils, mucosal lymphocytes, and epithelial cells are deeply involved and related each other to cause mucosal inflammation.

Meanwhile, rectal bleeding in infancy is related to lymphoid hyperplasia with eosinophil infiltration into the colonic mucosa facilitated by C-C motif ligand 11 (CCL11 known as eotaxin-1) and C-X-C motif chemokine ligand 13 (CXCL13). Rectal bleeding in infancy may not be simply caused by allergic reactions against specific antigens, but due to migrated lymphocytes to develop immunological tolerance; including IgA synthesizing, in the intestinal mucosa.

Key words:

C-X-C motif chemokine ligand 13 (CXCL13), food protein-induced proctocolitis (FPIP), high-affinity IgE receptor type I (FcεRI), IgA, mucosal damage, neonatal transient enterocolitis (NTEC)

1. Introduction

The physiological needs of nutrient absorption require that the gut has a large surface area, lined by a single layer of columnar epithelium. This means that the immune system of the gut is constantly bombarded by foreign proteins (food), while at the same time the immune system is specifically designed to react to the foreign proteins of pathogens. To get around this problem, the gut immune system has acquired mechanisms to avoid excessive reactions to foods, yet at the same time retain the ability to react to infectious agents. This system works efficiently in most individuals, but for an as yet undefined reason, some people react to food and other proteins as though they were pathogens, and induce chronic inflammation in the mucosa. Since food antigens are needed for nutrition, disease persists until the offending antigen is identified and eliminated from the diet.

The clinical features of inappropriate responses to food include vomiting, mal-absorption, diarrhea, bloody stool, eczema and a protean range of other symptoms. The adverse reactions caused by ingested foods are defined as food intolerance. Intolerance can be divided into two categories depending on whether or not they are immunologically mediated. This serves to exclude diseases where the pharmacologically effects of foods or additives cause adverse reactions.

Table 1 shows the classification of the food intolerance.[1] Non-immunologically mediated food intolerance includes the pharmacological effect of foods containing caffeine, alcohol, and toxins. It also includes primary and secondary metabolic disorders. Lack of lactase due to loss of microvilli during inflammation of intestine is included here. Idiosyncratic reactions, such as irritable bowel syndrome, may also be included but the mechanism of intolerance, if present, is obscure.

There are cases where immunological reactions overlap with non-immunological processes, for instance lactose intolerance in cow's milk allergy or inflammatory bowel disease. When we consider treatments, therefore, we have to be aware that both mechanisms may co-exist and exacerbate symptoms in a patient suffering from food intolerance.

2. Historical background of food intolerance

Adverse reactions to food were first reported more than 2000 years ago by Hippocrates. He had noted that gastric upset and urticaria could be induced by ingesting cow's milk. In modern times, Samuel Gee first described celiac disease in 1888, although it was a further 65 years before Dicke discovered the disease was induced by gluten.[2] Early in the 20th century, as the number of babies raised on formula milk increased, an increased incidence of eczema[3] and mortality due to gastroenteritis and pneumonia was seen.[4,5]

There is a need to prove that a certain food protein actually causes the adverse reactions, as opposed to another co-incident event. Thus, challenge tests were introduced in cow's milk intolerant patients.[6] This procedure of eliminating and giving suspected food protein directly to patients in a blinded fashion allows accurate definition of the actual food antigen that introduces clinical symptoms. Similar criteria were also used in soya protein intolerance.[7]

The discovery of immunoglobulin E (IgE) provided the mechanistic basis for immediate type hypersensitivity reactions to antigens, including foods.[8] The subsequent discovery of the role of T cells in delayed type hypersensitivity gave another pathway by which the immune system could react to food antigens and causes disease, exemplified by the T cell responses to wheat which causes celiac disease.

3. Immunological mechanisms of food tolerance

The immune system of the gut is continually exposed to benign foreign proteins, including foods, and yet, at the same time, the immune system must be able to react to pathogenic foreign proteins. To address this dichotomy, the gut immune system has acquired mechanisms to avoid excessive reactions to foods, known as "tolerance". TGF-β is an important cytokine which mediates active suppression against orally administered antigen. It is released by regulatory T cells and macrophages. In studies of multiple sclerosis patients, feeding myelin basic protein (MBP) slightly ameliorated disease activity.[9] Short term cultures of blood lymphocytes from these patients showed an increased in frequency of MBP specific T cells that secreted TGF-β1.[10]

The immunological mechanisms of food tolerance/intolerance are well studied in ovalbumin (OVA)-specific T-cell receptor transgenic mice. Oral administration of OVA introduces tolerance into these mice. Feeding lower doses of OVA causes active suppression by inducing TGF-β, IL-4 and IL-10 producing T cells. At higher doses, clonal anergy occurs by deleting Th1 and Th2 cells by apoptosis, although TGF-β producing Th3 cells seen spared.[11] IFN-γ producing cells are also seen in Peyer's patches (PP) however this is transient. After feeding a high dose of OVA, systemic administrations of anti-IL-12 antibody inhibits IFN-γ production and

enhances production of TGF-β in PP. IL-4 is not required for this TGF-β production, however it is able to enhance TGF-β production.[12] In addition, administration of OVA with anti-IL-12 antibody induced Fas-mediated apoptosis of antigen-specific T cells in local and systemic lymphoid tissue.[13] These studies demonstrate that orally administered food antigens can evoke TGF-β producing T cells, mostly $CD4^+CD25^+$ regulatory T cells, that can actively suppresses immunological reactions, and inhibit antigen-specific Th1 cell responses in the PP. High doses of antigen also induces tolerance by clonal deletion of antigen-specific T cells via Fas-mediated apoptosis.

4. The mucosal barrier in infants

Ingested foods are normally digested into peptides and amino acids by digestive enzymes before being absorbed by gut epithelial cells. Amino acids do not induce immunological reactions because they are too small to be recognized by antigen presenting cells (APC). However some proteins are poorly digested in the intestinal lumen, and indeed intact macromolecules can be detected as allergic reactions after feeding protein antigens. Immunological and non-immunological mechanisms control the penetration of these undigested proteins. For instance, mucus provides the intestinal epithelium with a mechanical (covering) and a chemical (washing) protection as well as an immunological protection involving secretory IgA (s-IgA). s-IgA is the predominant Ig class found in human maternal milk and maternal milk IgA compensates for the IgA deficiency of the newborn infant. s-IgA binds to antigens in the mucus layer and also regulates immune response to dietary antigens. Despite this however, macromolecules do cross the gut epithelium and can be recognized by the immune system.

In infants non-specific defenses in the gut may be compromised.[14] First, both intracellular and paracellular transport of macromolecules through epithelium is increased.[15] Second, insufficient secretion of digestive enzymes causes reduced digestion and increases the amounts of undigested proteins in the intestinal lumen. Third, the production and secretion of IgA is low. Fourth, infants often suffer gastrointestinal infections, which damage the epithelium and allow permeation of proteins into the lamina propria. Along these same lines, frequent infections activate mucosal immune cells and induce further reactions; such as induction of Th1 type cells and enhances the expression of MHC class-II molecules on intestinal epithelial cells. Hence, food intolerance can be a feature of the post-enteritis syndrome.

5. Mechanisms of food intolerance (Figure 1)

Type I allergy - Mast cells There is an increase in the number of IgE-bearing mast cells in the intestinal mucosa and submucosa of food allergic patients[16,17] and in mouse models.[18] The specific IgE bound to high-affinity IgE receptors on the surface of mast cells becomes cross-linked upon exposure to allergens, and activates mast cells. These mast cells release mediators such as histamine, leukotriene (LT)B_4, LTC_4, tumor necrosis factor (TNF)-α, IL-4, and other cytokines. Significant elevation of serum histamine,[19,20] LTB_4, LTC_4,[18] TNF-α concentrations are regularly observed after antigen challenge.[21,22] It is likely that these mediators induce a local increase in vascular permeability,[23] lymph duct dilatation and increased mucus production. They also cause watery diarrhea, resulting in blood hypocirculation and anaphylactic shock.

Type I allergy - Eosinophils Eosinophils are also important in Type I allergy. Eosinophil migration into the mucosa is often seen in patient with food allergic colitis, and typically presents in children with an atopic family

history, before the age of two years. Eosinophils migrate into the gut, responding to eosinophil chemotactic factors, such as eotaxin, platelet activating factor, histamine, and LTB_4, mostly derived from mast cells. The eosinophils also participate in IgE-mediated inflammation directly since they have low-affinity receptors for IgE.[24] Eosinophils liberate an abundance of highly cytotoxic and pro-inflammatory mediators, such as major basic protein, eosinophil cationic protein, and eosinophil peroxidase.[20,25] These mediators may disrupt mucosal surface and contribute to the later phase of reactions.

Type IV allergy - Cell mediated reactions Mucosal damage, such as villous atrophy and crypt hyperplasia is seen in biopsy specimens taken from patients with food protein-induced enteropathy. Similar pathological findings are also seen in patients suffering from persistent diarrhea, dehydration, malnutrition, and failure to thrive.[26,27] Since the mucosal infiltrate consists mainly of intraepithelial and lamina propria lymphocytes, but not of mast cells and eosinophils, cell-mediated responses are considered to be involved in mucosal damage in these patients. The relationship between cell-mediated reactions and their effects on intestinal morphology have been investigated in detail during graft versus host reactions,[28,29] which shows a striking morphological similarity to the mucosal damage seen in food protein-induced enteropathy. Direct evidence for an important T-cell mediated process in the pathogenesis of the flat mucosal lesion has been shown in other *ex vivo* model systems.[30] The same group also reported that as a result of an uncontrolled activation of T-cells, increased synthesis of matrix metalloproteinases (MMP)[31] and keratinocyte growth factor (KGF)[32] contribute to the gut damage. It is considered that MMPs and KGF may be directly involved in mucosal damage since they cause the degradation of mucosa and the crypt hyperplasia by stimulating epithelial cell proliferation.

Mixed reactions Could IgE-mediated reactions be involved in prolonged symptoms by chronic antigen exposure? This may be highly relevant to the clinical situation, because even Type I allergic patients often suffer from frequent and prolonged allergic responses as a result of repeated antigen exposure. Hence, we have shown that not only mast cells but also eosinophils and lymphocytes infiltrate into the intestinal mucosa upon chronic antigen exposure in antigen-specific IgE-mediated mouse model.[33] Cell adhesion molecule expression on endothelial cells is up-regulated as a result of continuous stimulation with chemical mediators derived from mast cells, especially TNF-α.[34,35] This enhancement leads to increased infiltration of lymphocytes into the mucosa. These lymphocytes that have migrated, especially Th1 cells, produce IFN-γ and up-regulate antigen presentation by promoting MHC-class II molecule expression on APC in the intestine.[36] This further promotes cell-mediated reactions against food antigen.

Epithelial cells The primary function of the intestinal epithelial cells is to absorb nutrients while providing a physical and immunological barrier to the external world. Nevertheless epithelial cells are in direct contact with the intestinal milieu and they produce several chemokines influenced by the luminal contents that include bacteria, bacterial products, and chemical stimuli.[37-42] By using the transgenic technique, we have described that neutrophil and lymphocyte infiltration into the mucosa due to a single chemokine, macrophage inflammatory protein (MIP)-2, expressed by the epithelial cells.[43] Furthermore, the finding of the greatly increased neutrophil recruitment in MIP-2 transgenic mice by stimulation with dextran sulfate sodium (DSS), which may produce

more MIP-2 in the small intestine, than do wild-type mice (Figure 2) suggest that chemokine production from the epithelium is an important mechanism of DSS induced mucosal inflammation. DSS may therefore stimulate intestinal epithelial cells and enhance the chemokine secretion to further increase neutrophil invasion.[44]

6. Rectal bleeding in infancy/ NTEC / FPIP / CXCL13

Eosinophilia is relatively common in newborns and is caused by an increased rate of hemocytopoiesis, especially in pre-term infants, although most cases are symptomless. However, a closer examination of infants with bloody stool can often reveal abnormally elevated eosinophil counts. Since we have experienced several cases of neonates who showed rectal bleeding with massive eosinophilia on the first day of life before enteral feeding, and since these changes are antigen non-specific and are only observed transiently in neonates, we have termed this disease "neonatal transient eosinophilic colitis (NTEC)," which represents a new category of eosinophilic gastroenteritis.[45] In these infants, we performed rectosigmoidoscopy to confirm the cause of rectal bleeding and found nodular lymphoid hyperplasia with a pale mucosal surface and massive bleeding around the recto-sigmoid colon with diffuse eosinophil infiltration in the lamina propria, which is similar to the signs of food protein-induced proctocolitis (FPIP).

To investigate the outcome and pathogenesis of rectal bleeding in infancy, especially when concomitant with FPIP and NTEC, 22 neonates with rectal bleeding with FPIP and NTEC from January 2008 to June 2012 were enrolled and their clinical course and mechanisms of inflammation were examined by microarray analysis.[46] FPIP in infancy and NTEC are similar diseases and that IL-6, C-C motif ligand 11 (CCL11 known as eotaxin-1), and C-X-C motif chemokine ligand 13 (CXCL13) may play a major role in the pathogenesis of rectal bleeding (Figure 3). Although the involvement of allergic reaction is possible,[47] cow's milk allergy was not a common outcome after 1 year of follow-up.

CXCL13 As explained before, synthesizing IgA molecules targeting multiple antigens is an important task for the mucosal immune systems of neonates. IgA is mainly synthesized in the lymphoid follicles of the intestine, and the enhanced CXCL13 and CXCR5 (receptor for CXCL13) expression observed in neonates is convenient for IgA synthesis because CXCL13 is a chemokine related to lymphoid follicle formation.[48] The immunohistochemical analysis revealed that infiltration by IgA-bearing cells was pronounced in FPIP (Figure 4) and NTEC, possibly suggesting that the enhanced immunological reaction against food antigen, including producing IgA, is one of the major elements of the pathogenesis of rectal bleeding in infants (RBI). Because lymphoid hyperplasia is one of the characteristic findings in RBI[49] and because IgA synthesis is predominant after birth, these changes can be favorable immunological findings in infants, including those with RBI.

7. Symptoms

Although food antigens are encountered through the gut, the symptoms of food intolerance are often seen in skin and mucosa of eyes, nose, respiratory system, causing eczema, erythema, itchiness, wheezing, and respiratory distress. The common digestive symptoms of food intolerance are diarrhea, bloody stool, vomiting, and nausea. Edema and ulceration of oral cavity as well as constipation are seen in some cases.[50,51] Digestive

symptoms are often severe in infants and young children. Respiratory and dermal symptoms are also seen, but are more frequent in older children.

These symptoms, observed within minutes to an hour after antigen ingestion, are due to antigen specific IgE-mediated reactions. These symptoms often start from the location where antigen is encountered, and sometimes develop to severe systemic reactions. In the intestine, biopsy specimens reveal mucosal edema and mast cell and eosinophil infiltration.[52] IgE-mediated reaction in intestine cause secretary diarrhea and bloody diarrhea in patients with food allergic colitis. Diagnosis can be ascertained by the patient's past history, skin tests, and the presence of food-specific serum IgE antibodies, and oral food challenge test. Oral food challenge is necessary because the accuracy of antigen specific-IgE level and skin tests is generally unsatisfactory. However, challenge tests can be dangerous and induce shock, therefore should be performed under intensive surveillance.

On the other hand, cell-mediated reactions are involved in the prolonged symptoms, such as persistent diarrhea, dehydration, and failure to thrive. These symptoms are caused by mucosal damage, evident as villous atrophy and crypt hyperplasia.[26,27] Patients who suffered from cell-mediated reactions therefore show persistent diarrhea with dehydration, malnutrition, and failure to thrive after antigen exposure. Prolonged exposure to antigen may eventually result in anemia, hypo-proteinemia and hypo-circulation.

8. Treatment

Identification and elimination of the proteins that cause the adverse reaction improves the condition of these patients. In immunological food intolerance, hydrolysed cow's milk, soya, and gluten, are available. In Type I allergy, nutritional status and the level of hydration have to be considered. Elimination of the offending food from the diet should prevent further malnutrition and dehydration. Hypolactasia may be seen in prolonged diarrhea with brush border damage, so that substituting lactose with another sugar such as glucose is necessary. However continuing elimination diet is not the fundamental treatment. Eliminating antigenic food for a few weeks can retain proper digestive function and by administrating antigenic foods from small amount may be able to induce tolerance against antigenic food proteins, which is known as oral tolerance induction therapy.[53]

There are multiple steps that can be taken in intervention against immunological food intolerance. One potentially effective approach is to inhibit IgE-binding to the high-affinity IgE receptor (FcεRI) on mast cells to abrogate mast cell activation.[54] It has been demonstrated that the soluble form of human FcεRIα chain (sFcεRIα) suppresses the allergic response in passively sensitized mice.[18] Allergic patients suffer from frequent IgE-mediated responses as a result of repeated antigen exposure, and these allergic reactions could be completely blocked by continuous administration of sFcεRIα since it can efficiently trap free IgE in the vicinity of mast cells before binding of the IgE to the cell surface FcεRI. An alternative approach is to use a monoclonal anti-IgE antibody, which binds IgE on the mast cell membrane and blocks allergen-induced mast cell degranulation.[55] Meanwhile, immune-modulatory effects of probiotics and omega-3 fatty acids have been investigated and are considered to be beneficial for inducing tolerance and protecting intestine from inflammation.[56,57]

9. Probiotics and tolerance

Probiotics are useful for regulating inflammation as well as allergic reactions. Although lactobacilli and bifidobacteria are often used as probiotics, their functions seem to be different. *Nonaka et al.* suggested that

lactobacilli modulate the Th1/Th2 immune balance toward Th1 reactions.[58] *Shima et al.* reported that the *Lactobacillus casei* strain Shirota enhances the expression of genes involved in defense/ immune functions and in lipid metabolism, whereas the *B. breve* strain Yakult down-regulates the expression of many genes.[59] *Hoarau et al.* suggested that *B. breve* can induce dendritic cell maturation through TLR2, with production of IL-10 regulating excessive Th1 responses as well as Th2 polarization.[60] Our findings support a possible mechanism for *B. breve* action in which it modulates inflammation by down-regulating the expression of inflammatory molecules (lipoprotein lipase, glutathione peroxidase 2, and lipopolysaccharide binding protein) during the newborn period. It would also promote tolerance by up-regulating the expression of CD3 but not co-stimulatory molecules during the weaning period.[56] Further study is needed to determine the precise effects of *B. breve* in immune reactions during early infancy.

In the clinical trials, *Rautava et al.* reported that administrating probiotics during pregnancy and breast-feeding can increase the concentration of TGF-β_2 in breast milk and provides protection against atopic eczema during the first 2 years of life.[61] *Arslanoglu et al.* reported that early dietary intervention with a mixture of prebiotics oligosaccharides reduces the incidence of allergic manifestations and infections during the first 2 years of life.[62] *Kim et al.* also reported that mixture of probiotics (*Bifidobacterium bifidum, Bifidobacterium lactis, Lactobacillus acidophilus*) have beneficial effect to prevent development of eczema in infants at high risk during their first year of life confirmed by double-blind, randomaized, placebo-controlled trial.[63] It is considered that immune-modulatory effects of probiotics are depended on the strain, amount, and timing of probiotics used in each study. A possible beneficial effect of probiotics should be explored in future randomized- controlled trials.

10. Conclusion

Studies of cytokine and their signaling network leads us deeper understand of the mechanisms of food intolerance and mucosal inflammation. By identifying the mechanisms of food intolerance and the cells responsible for causing the mucosal inflammation; such as mast cells, eosinophils, Th1 and Th2 cells, it will make the treatment efficient. Hopefully, these studies will give us an idea of how we can establish immunological tolerance in patients with food intolerance, thereby bypassing the need for eliminating diets.

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Figure legends

Figure 1 Mechanisms of food allergy in the intestine.

Antigen and its specific IgE bound to high-affinity IgE receptors activate mast cells. Histamine, eotaxin, leukotriene (LT)B_4, LTC_4, tumor necrosis factor (TNF)-α, IL-4, and other chemical mediators are released and induce a local increase in vascular permeability and mucus production, and elicit secretary diarrhea.

Eosinophils migrate into the gut, responding to eosinophil chemotactic factors, such as eotaxin, platelet activating factor, histamine, LTB_4 and etc. Eosinophils release major basic protein, eosinophil cationic protein, and eosinophil peroxidase, which are highly cytotoxic pro-inflammatory mediators and may disrupt mucosal surface and contribute to the later phase of reactions.

TNF-α released from mast cells enhances the expression of adhesion molecules on endothelial cells and leads lymphocyte infiltration into the mucosa. This may promote further cell-mediated reactions against food antigen in the intestine.

As a result of an uncontrolled activation of Th1 cells, increased synthesis of Th1 cytokines stimulates macrophages, and stromal cells. They may contribute to mucosal damage, such as villous atrophy and crypt hyperplasia, by releasing keratinocyte growth factor and matrix metalloproteinases. Meanwhile activated Th2 cells promote antigen-specific IgE and induce further allergic reactions to the antigen.

Abbreviations: eosinophil cationic protein (ECP), eosinophil peroxidase (EPO), high-affinity IgE receptors (FcεRIα), intercellular adhesion molecule-1 (ICAM-1), keratinocyte growth factor (KGF), leukotriene (LT), major basic protein (MBP), matrix metalloproteinases (MMP), mucosal addressin cell adhesion molecule-1 (MAdCAM-1), platelet-activating factor (PAF), tumor necrosis factor (TNF)

Figure 2 Epithelial cells in the inflammation.

a. Neutrophil recruitment in the small intestine from wild-type and macrophage inflammatory protein (MIP)-2 transgenic mice with or without 2.5% dextran sulfate sodium (DSS) treatment. Neutrophil recruitment was greater in MIP-2 transgenic mice than in wild-type mice after DSS treatment. Neutrophils were stained by myeloperoxidase (MPO) using the Hanker Yates reaction. Magnification×100.

b. Neutrophil recruitment was much greater in MIP-2 transgenic mice than in wild-type mice both in the small intestine and colon after 2.5% DSS treatment. Neutrophil recruitment was demonstrated by total MPO activity. MPO was extracted from the whole small intestine and colon with 0.5% hexadecyltrimethylammonium bromide. Open columns show total MPO activity per unit weight of tissue derived from wild-type mice, and closed columns show those of MIP-2 transgenic mice. Data are presented as the mean±SD. *$p<0.01$.

Figure 3 Rectal bleeding in infancy.

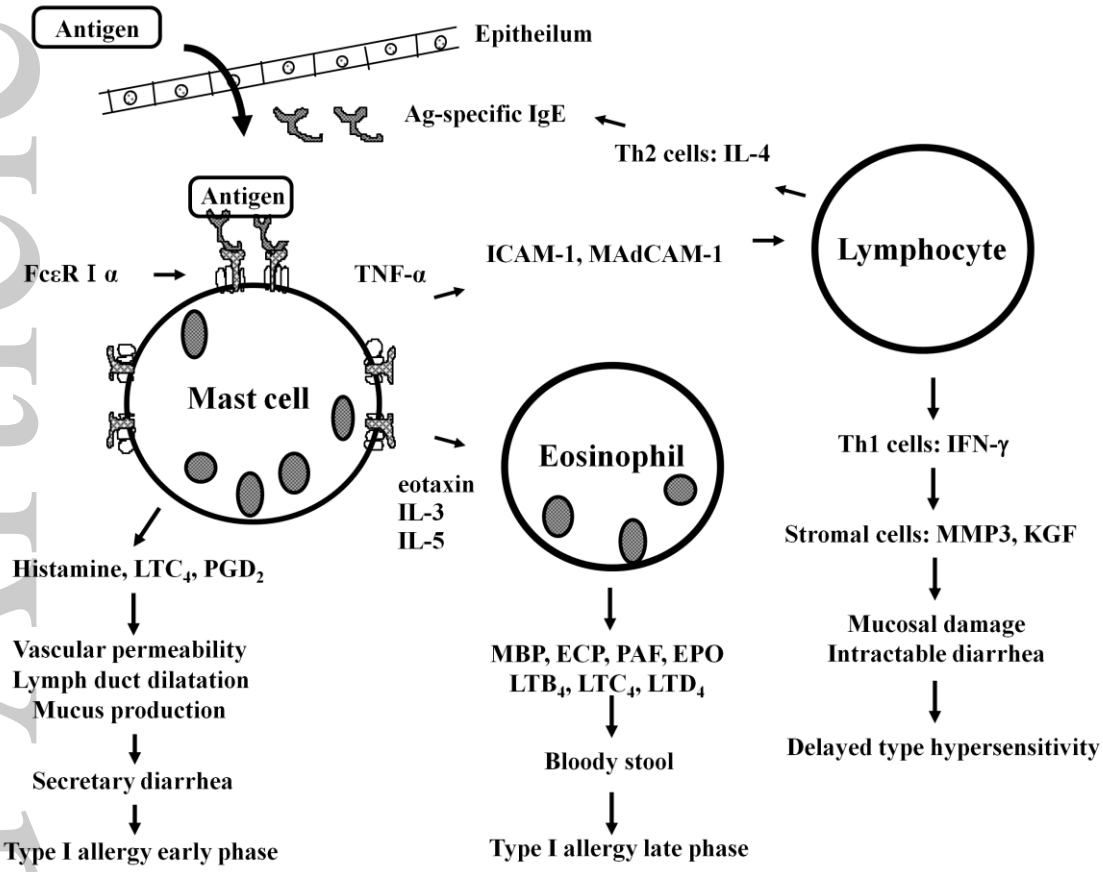
Rectosigmoidoscopy revealed nodular lymphoid hyperplasia with a pale mucosal surface and massive bleeding around the recto-sigmoid colon with diffuse eosinophil infiltration in the lamina propria. Microarray analysis revealed the enhanced expression of C-C motif ligand 11 (CCL11 known as eotaxin-1) and C-X-C motif chemokine ligand 13 (CXCL13) in these mucosa. Expression of CCL11 was more dominant in neonatal transient eosinophilic colitis (NTEC) where the expression of CXCL13 was more dominant in food protein-induced proctocolitis (FPIP).

Figure 4 Histological image of a representative sample from infants with food-protein induced proctocolitis (FPIP). The immunohistochemical analysis was performed using anti- immunoglobulin (Ig)A antibody. IgA-positive cells were present in the lamina propria. Magnification ×400.

Table 1 Classification of food intolerance

1. Non-immunological reactions
   a) Predictable reactions
      Pharmacological effects;
      Lectins, protease inhibitors, caffeine, salt, vasoactive amines, alcohol, etc.
      Bacterial effects; Enterotoxines and other toxins
   b) Adverse food reactions associated with metabolic defects
      Hypolactasia; Primary and secondary
      (IBD, enterocolitis, food intolerance etc.)
      Primary enzyme deficiencies;
      Sucrase-isomaltase, trehalase, trypsinogen, lipase, G6PD, etc.
   c) Idiosyncratic reactions
      Irritable bowel syndrome, Non-IgE mast cell degranulation, etc.

2. Immunological reactions
   a) IgE-mediated reactions; Mast cells and eosinophils
   b) Antibody-dependent cytotoxic reactions
   c) Immune-complex mediated reactions
   d) Cell-mediated reactions
   e) Mixed-reactions



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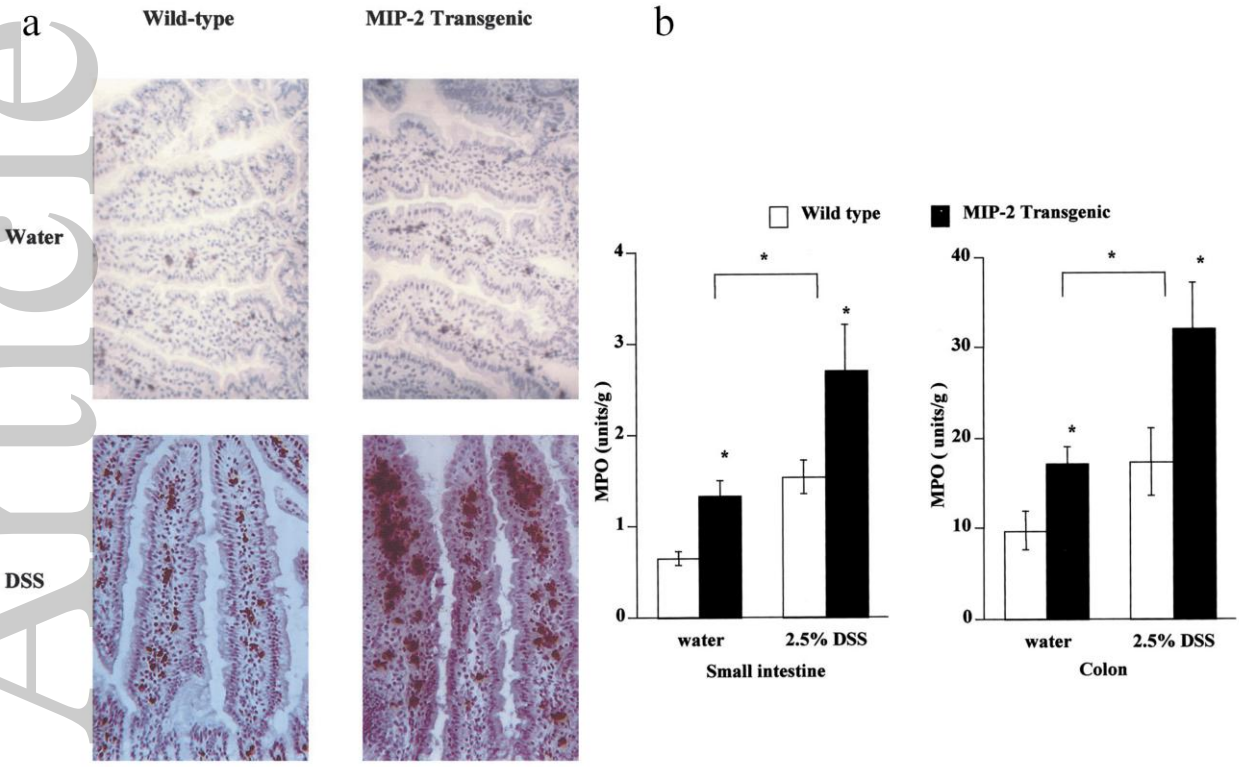

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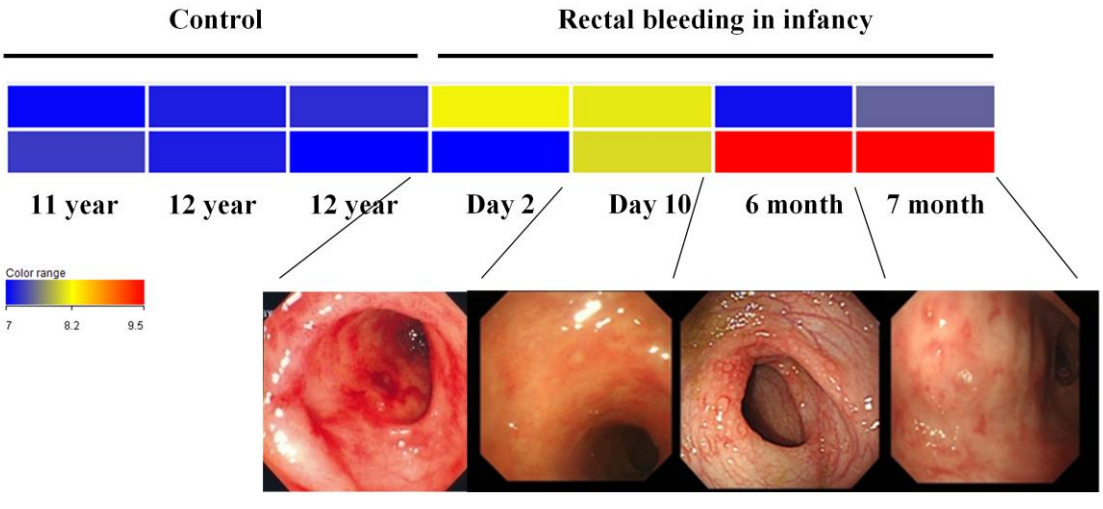

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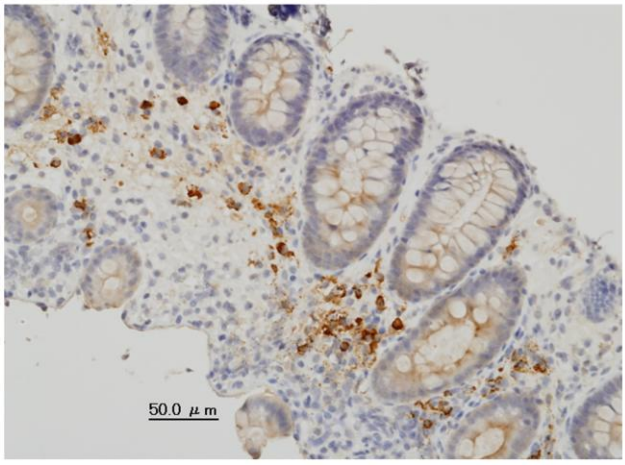

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